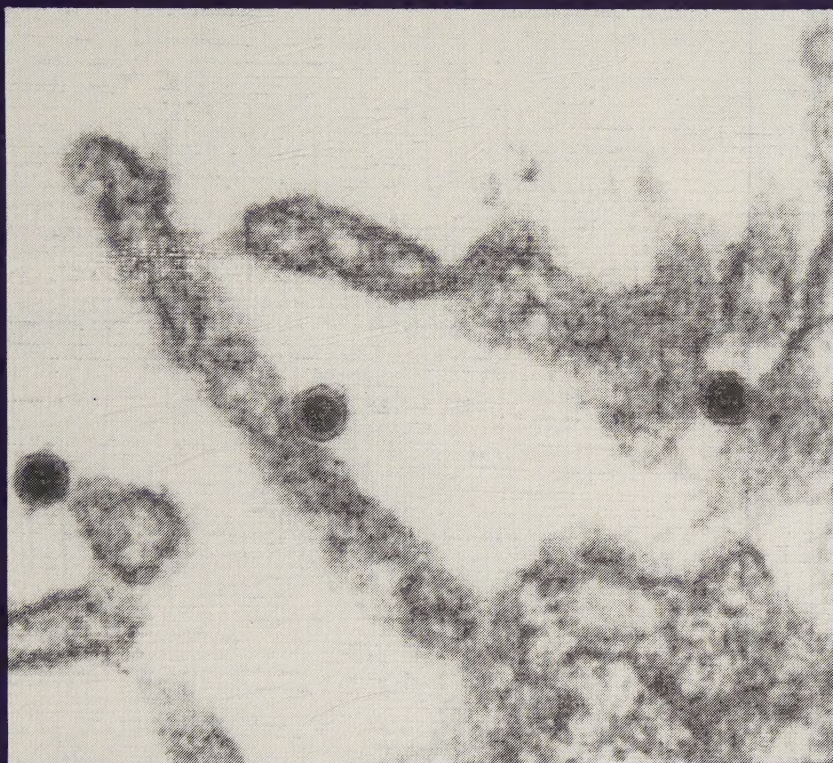


Early interaction
between adenovirus type 2 and HeLa cells



Robert Persson



Lund 1984



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From the Department of Microbiology, University of Lund,
Lund, Sweden

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by

Robert Persson

Lund 1984

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Cover shows adenovirus type 2 attached to microvilli on a HeLa cell.

C
Robert Persson

Francois Jacob -

Membrane recognition events may be explained by the properties of their components but can not be deduced from them.

(a paraphrase from The Logic of Living Systems, 1974)

To Christine
my mother
the memory of my father

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This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals.

- I. Svensson, U., R. Persson, and E. Everitt. 1981. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. *Journal of Virology* 38:70-81.
- II. Persson, R., U. Svensson, and E. Everitt. 1983. Virus receptor interaction in the adenovirus system. II. Capping and cooperative binding of virions on HeLa cells. *Journal of Virology* 46:956-963.
- III. Svensson, U., and R. Persson. 1984. Entry of adenovirus 2 into HeLa cells. *Journal of Virology* 51:687-694.
- IV. Persson, R., C. Wohlfart, U. Svensson, and E. Everitt. Virus-receptor interaction in the adenovirus system. III. Characterization of the positive cooperative binding of virions on HeLa cells. Submitted to *Journal of Virology*.
- V. Persson, R. Virus-receptor interaction in the adenovirus system. IV. Immunochemical identification of a cellular receptor protein. Submitted to *Journal of Virology*.

SUMMARY

The attachment of adenovirus type 2 (Ad2) to specific receptor sites on the HeLa cell plasma membrane was investigated. A membrane protein with affinity for Ad2 virions was isolated by affinity chromatography on immobilized glutaraldehyde-fixed virus particles. The protein contains acetylated glucosaminyl residues and has an apparent polypeptide molecular weight of 40,000 to 42,000. A similar and probably identical protein was identified from immunochemical studies with an antiserum against plasma membranes which inhibit the virus attachment. Therefore it is concluded that this protein constitutes, or is a part of, the cellular receptor sites for Ad2 on HeLa cells.

Analyses of the virus attachment to cells revealed that there are around 5,000 receptor sites on HeLa cells and the association constant of the interaction is $1.5 \times 10^{10} (\text{M}^{-1})$ per site. The attachment exhibited positive cooperativity at 37°C which was inhibited at low temperatures, by prefixation of cells with glutaraldehyde or by reagents inhibiting the clustering of ligand-receptor complexes in the plasma membrane. The positive cooperative binding was also dependent on the multivalency of the virion attachment protein on the actual virus particles. The natural labilization of attached virions, which renders the viral genome sensitive to DNase treatment, was likewise dependent on the temperature and a "fluid" membrane. This destabilization of virions was shown to occur at the outside of the plasma membrane and it was essential for the internalization of the attached virions.

Taken together, the results of this investigation demonstrate how adenovirus type 2 virions attach to specific receptor sites on the HeLa cell. The requirement for diffusion of virus-receptor site complexes in the plasma membrane to prepare the virus particles for the subsequent internalization, is stressed.

INTRODUCTION

During productive replication the animal virus undergoes six discrete stages. The first stage includes attachment to the cell surface, the passage through the plasma membrane and uncoating, which prepares the virus for nucleic acid replication and expression of the viral genes. Thus, the second, third and fourth stages are comprised of nucleic acid replicational events, transcription and translation of the genome. The fifth and sixth stages - assembly and release of progeny particles - are in some virus systems indistinguishable from each other. The study of the initial stages has been overshadowed by massive interest in virus-directed macromolecular synthesis. However, research in the area of early interactions between animal viruses and cells has become more intense during the past six to eight years. At present a better understanding in several virus systems of these stages which are the absolute prerequisite for a successful infection has become available.

Viruses adsorb nonspecifically to different types of nonbiological surfaces, such as glass, nitrocellulose, aluminium, and carbon (5, 113). These interactions may give some information about the significance of certain physico-chemical parameters, e.g. the rate at which a virus particle arrives at a surface can be predicted from the theory of Brownian motion. On the other hand, the attachment of viruses to specific sites on the plasma membrane of host cells, which subsequently leads to a productive infection, reveals the biological importance of this interaction.

The fluid lipid matrix of the plasma membrane harbors proteins (103), which are responsible for most of the functional properties of the membrane. These include reception and transduction of extracellular signals and transportation of small molecules from the outside of the cell to the inside, or vice versa (77, 104). It is very important in these processes to have a membrane where the components can migrate laterally in the plane of the membrane, enabling them to come together and interact. Thus, the importance of lateral migration and clustering of ligand-receptor complexes has been demonstrated as an integral part of the mechanism leading to the cellular activation in response to peptide hormones (48, 99, 100). Likewise, Semliki Forest virus attached to

specific binding sites on the microvilli on BHK cells, seem to migrate on the cell surface to finally reach specialized structures, referred to as coated pits, before the step of internalization (28).

The aim of this investigation was to identify and isolate the protein(s) constituting the cellular receptor sites for adenovirus type 2 on HeLa cells and to characterize the attachment mechanism of this virus.

Cellular binding sites for animal viruses

The extracellular interaction between a virus and the host cell is generally very strong (see next section) and involves particular structures of the virus particles, but also in many cases specific sites on the cell surface. Even though the cellular binding sites are specific for a certain virus, it is unlikely that they are synthesized for the a priori function of being the structures responsible of future infection by that virus. Therefore the term "virus receptor" may be considered as a misnomer for these structures, and instead the nomenclature proposed by Lonberg-Holm (61) should be applied. This terminology contains the following items: Virus attachment protein(s) (VAP) are virion structure(s) which recognize a cellular receptor; and cellular receptor units (CRU) are cellular molecules recognizing one VAP; and cellular receptor sites (CRS), which are cellular structures composed of one or more CRUs involved in the binding of a virus particle. The VAP structure has been identified and characterized for many virus systems (11), while the CRU/CRS in the majority of virus-cell systems is unknown. This may be because any component of the cell plasma membrane - lipid, carbohydrate or protein - can serve as a binding site, alone or in any possible quantitative or qualitative combination.

The lipid moiety: Because the lipid components of the plasma membrane of many cell types are similar, viruses which recognize lipids as receptor sites might be expected to exhibit a broad host range. This is also the case for vesicular stomatitis virus and some members of the Togaviridae family. These viruses attach specifically to certain phospholipids on the plasma membrane (19, 101). Even though the Sendai virus binds to artificial liposomes composed of only phospholipids and chole-

terol (75), it appears as if specific gangliosides function as cellular receptor sites for this virus in the permissive host cells (67). It was furthermore shown, by Markwell et al (67), that the terminal N-neuraminic acid (NANA) residues on the gangliosides play a vital role in the Sendai virus-cell interaction.

The plasma membrane carbohydrates: Possibly the most thoroughly investigated interactions between animal viruses and cells involve the NANA-residues. This acid is normally positioned at the terminus of an oligosaccharide, which in turn may be linked either to a glycolipid (as mentioned above) or to a glycoprotein (51). It is generally accepted that NANA-residues are required for the cellular attachment of orthomyxo- and paramyxoviruses, since neuraminidase treatment of cells to various degrees impairs subsequent virus attachment (26, 37, 111). Other viruses that also recognize NANA-containing receptor sites for attachment are; cardioviruses (49, 116), polyomaviruses (27, 76), and members among the reoviruses (22). All these viruses have the ability to agglutinate red blood cells, but the interesting fact that neither free NANA-molecules (1, 123) nor oligosaccharides containing NANA-residues (23) inhibit virus hemagglutination has lead to the hypothesis that the NANA-residue must be relegated to an indirect role in the attachment process for these viruses. This hypothesis suggests that the NANA moieties interact with basic amino acids of the NANA-containing glycoprotein itself to confer a particular three-dimensional configuration (15, 59, 60). Consequently, both the carbohydrate and the protein components, in these instances, are necessary for the viral recognition of cellular sites.

Protein components of the plasma membrane: Some viruses have been shown to attach to cellular receptor sites of a proteinaceous nature, since it is possible to remove these binding sites with different proteolytic enzymes (62). Because many of the membrane proteins on the non-cytoplasmic surface are glycosylated (96), it is difficult to determine if the virus attachment is dependent on the protein structure, the carbohydrate structure, or both. Treatment with specific glycosylases or metabolic inhibitors interfering with the biosynthesis of saccharide-chains linked to proteins may be useful, provided that the protein configuration is unchanged. Even though the protein nature of some

cellular receptor sites is known, only a few CRSs have been characterized. For instance, acetylcholine receptors have been proposed to function as cellular receptor sites for rabies virus on mouse muscle cells (58). This conclusion was based on the observation that treatment of the cells with toxins (α -bungarotoxin and d-tubocurarine), which bind to the acetylcholine receptor, decreased subsequent virus attachment and replication in such cells. The similar cell surface distribution of attached viruses and acetylcholine antagonists also strengthened this assumption. In another example, the VAP of Semliki Forest virus (SFV) has been shown to recognize the major histocompatibility antigens (MHC) on human and murine cells. Antibodies against the MHC consequently inhibit the cellular attachment of constructed complexes of VAP (29). The attachment situation for the complete virion might however be somewhat different from that of isolated VAP structures, and indeed Oldstone et al (87) showed that SFV is capable of infecting cells which do not express the major histocompatibility antigens. Another possibility is that viruses might recognize different CRSs on two different cell types. However, certain cellular and tissue tropisms have been demonstrated, as exemplified in the Epstein-Barr virus system, where only B-lymphocytes and not T-lymphocytes display receptors for this virus (24, 46, 72). Furthermore, a tissue tropism in humans is well established for the picornaviruses (14).

Attachment of animal viruses to cells

When the binding of a virus to a specific site ("receptor") on a cell is analyzed, the data must be carefully interpreted. If, for instance, the ligand-receptor complex is internalized or if the ligand is modified in connection to the attachment, the obtained binding results might not be reflections of true attachment (4). Thus, the ligand binding must be studied at equilibrium, which of course is easier to achieve at temperatures around 4°C, where e.g. the internalization process is impaired. Ligand-cell interactions are complex and are not only involving the particular counterparts studied, but also adjacent components - such as lipids and membrane proteins on the cell surface and virus structural proteins - may indirectly be engaged. Furthermore, the binding sites are able to diffuse laterally and interact with each other thereby providing different degrees of cooperativity of the virus or the ligand binding

(78, 115). Positive cooperative binding refers to a situation where binding of the first ligand to one receptor site promotes the binding of subsequent ligands to other sites. On the other hand, negative cooperativity describes a condition when the binding at the first ligand impairs the interaction of the following ligands. This may be the consequence where binding is achieved to two or more classes of binding sites with different affinities for the ligands. Mathematical expressions for interactions between ligands and cells are in most cases adoptions from binding studies of small molecules (e.g. ions and oxygen) to proteins (34, 98) and also from enzyme kinetic analyses (57, 73).

If a cell (C, the macromolecule of the system), which is assumed to have n binding sites, reacts with a virus (V, the low-molecular weight component of the system) the binding of V can be expressed as:
 $\bar{v} = \frac{[V] \text{ (bound)}}{[C] \text{ (total)}}$. If there is a saturation level of the virus binding to the cell surface, a graphical representation of $\bar{v}$ versus $[V]$, will give a curve where the number of binding sites (n) is obtained when $\bar{v}$ approaches infinity. Provided the ligand binding at any site is independent of the occupation of other sites, and if all sites are the same, the fraction bound ($\bar{v}$) at equilibrium may be expressed as:
 $\bar{v} = \frac{n \cdot K \cdot [V]}{1 + K \cdot [V]}$, where K is the association constant. Because of the complexity of the interactions and the usage of concentrations in the expression instead of activity coefficients, the association constant obtained must be regarded as a practical one and not as a true thermodynamic equilibrium constant (115). The latter expression can further be rewritten into two forms, I and II, useful for graphical tests and for the determination of n and K .

$$\frac{1}{\bar{v}} = \frac{1}{[V]} \cdot \frac{1}{n \cdot K} + \frac{1}{n} \quad (\text{I})$$

In a double-reciprocal plot as described by Lineweaver and Burke (57), $\bar{v}^{-1}$ is plotted versus $[V]^{-1}$ (Figure 1A), the slope of the curve obtained corresponds to $\frac{1}{n \cdot K}$, and $\frac{1}{n}$ is derived from the intercept on the abscissa. Cooperative binding situations are illustrated in the figure by the concave upward line (positive cooperativity) and the convex upward line (negative c.). The straight line represents non-cooperative binding of a ligand.

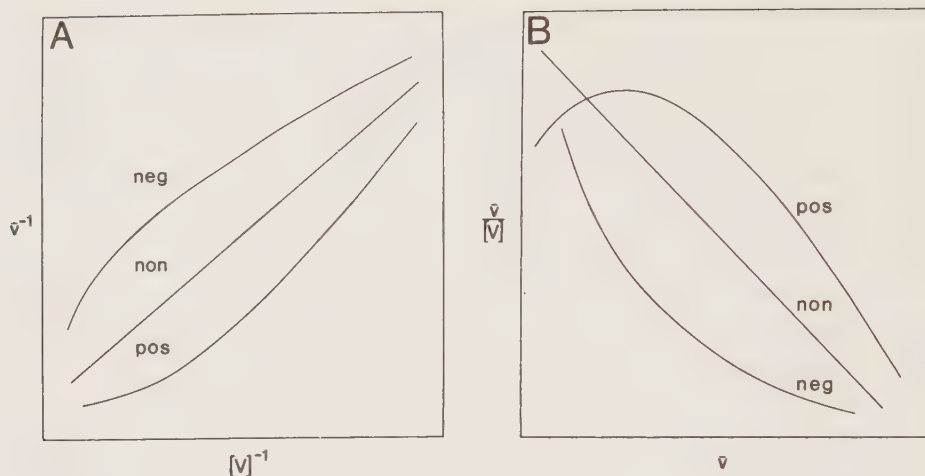


Figure 1. A graphical representation of ligand binding data demonstrating positive cooperativity (pos), non-cooperativity (non), and negative cooperativity (neg). In panel A. a double-reciprocal plot, and in B. a Scatchard plot.

$$\frac{\bar{v}}{[V]} = K \cdot n - K \cdot \bar{v} \quad (\text{II})$$

A plot of $\frac{\bar{v}}{[V]}$ versus $\bar{v}$ (Figure 1B), as described by Scatchard (98), reveals a curve with the slope yielding $-K$, and the intercept of the abscissa corresponding to n . Here, positive cooperativity is indicated by a maximum of the curve, while a downward concave curvature represents negative cooperativity. A situation of non-cooperative binding is illustrated by the straight line. The extent of curvature in both of the illustrated plots, is a measure of the degree of cooperativity. A most convenient means to quantitatively express the degree of cooperativity, is to plot $\log \frac{\bar{v}}{n-\bar{v}}$ versus $\log [V]$ (Fig. 2) and to determine the slope of this curve. Such a graph is called a Hill plot (34) and the value of the slope - the Hill coefficient (N_H). This value will take into account both the number of ligand molecules in the interacting unit and the degree of interaction between them. A value above unity is indicative of a positive cooperative binding and a value below unity represents a negative ditto. Non-cooperative binding will yield an N_H -value of 1.

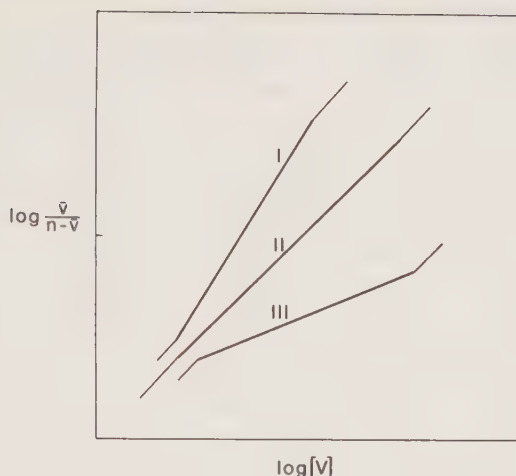


Figure 2. A Hill plot illustrating ligand binding data with different degrees of cooperativity. Curve I, positive cooperativity ($N_H=1.6$); curve II, non-cooperativity with a coefficient value of 1.0; and curve III, negative cooperativity ($N_H=0.5$). The thicker part of a line denotes a region in which experimental results often appear linear.

Compared to enzymatic reactions, with association constants around 10^3 to 2×10^5 (M^{-1}) (41), many specific interactions between ligands and cells are much stronger. Association constants of 10^7 to 10^{13} are often obtained for the binding of different ligands (e.g. antibodies, toxins, viruses) to relevant cells (41).

A multivalency of the ligand, with respect to the binding property, may in most cases explain the strong association to cells. For instance, the association constant calculated on a per cell basis for rabbit anti-human red blood cell antibodies interacting with red cells is 400 times higher than for the corresponding monovalent Fab-fragments (25).

Studies on the attachment of different animal viruses to various cell types have revealed association constants from 10^8 to 10^{11} (M^{-1}) and the estimated number of receptor sites per cell ranged between 4×10^3 and 8×10^4 (16, 18, 70, 102). The binding of reovirus 1 and 3 to L-cells (a murine cell line) was shown to be noncooperative and the interaction

between vesicular stomatitis viruses and Vero cells (an african green monkey kidney cell line) exhibited negative cooperativity, while the Semliki Forest virus binding was either non- or negatively cooperative depending on the recipient type of cell (16, 18, 102). In all of the three investigations the virus attachment was studied at low temperatures (i.e. 4 or 5 °C). To learn if the cooperative binding of these viruses is temperature dependent, attachment studies at an elevated temperature - relevant for natural infection - are required.

In a totally different system, Prujansky et al (94) showed that binding of multivalent lectins to lymphocytes at room temperature exhibited positive cooperativity, whereas the binding of their monovalent counterparts did not. In addition, the mitogenic response of the lectin treatment was also dependent on the multivalency of the ligand. Therefore, these authors suggested that positive cooperativity, being a reflection of clustering of lectin-receptor complexes and conformational changes in the membrane structure, is a prerequisite for mitogenic stimulation (94).

The model system

Since the pioneer isolation of adenovirus in 1953 (97), more than 90 serotypes from different animals have been characterized (83). Two distinct genera within the family of Adenoviridae can be distinguished, namely, the mammalian adenoviruses with one antigenic determinant in common, and the avian adenoviruses (83). The human adenoviruses, of which at least 35 serotypes have been isolated (45, 105), are further divided with respect to their oncogenicity (38) or their capacity to agglutinate red blood cells from different animal species (95). The human adenoviruses of type 2 (Ad2) belong to the hemagglutinating subgroup III and agglutinate rat and human O red blood cells (118). Furthermore, this serotype exhibits no oncogenicity when injected into newborn hamsters, but has the capacity, in vitro, to transform cells that are nonpermissive for virion replication (68, 69).

The adenovirus particle is an icosahedron of 70 to 90 nm and is composed of 252 capsomers ((35) Fig. 3). Two hundred and forty of these capsomers, called hexons, make up the 20 triangular facets of the spherical polyhedral structure (21). The remaining 12 capsomers, called pentons, form the 12 apical structures (21). The penton is composed of a penton base, and a projecting shaft terminated by a knob; this projection is called the fiber (80, 114). Covered by the protein capsid is a nucleoid, consisting of double-stranded DNA associated with internal basic proteins (20). The length of the linear Ad2 genome is 35,937 nucleotides as determined by DNA sequence analysis (117).

The hexon protein, with a native molecular weight of 310,000 to 360,000 (17), has been shown to consist of three identical polypeptide monomers with a molecular weight of 95,000 to 120,000 (12, 36). The hexon carries the group-specific determinant common to all mammalian adenoviruses (53). The length of the projecting fiber protein differs among the serotypes. Adenoviruses belonging to the hemagglutinating subgroup III (e.g. Ad2) have the longest fibers, 25 to 30 nm (82). The molecular weight of the native fiber protein from Ad2 has been estimated to be between 160,000 and 200,000 (13, 108). Because the molecular weight of the subunit constituting the fiber is close to 62,000 (33), it implies that the native fiber is built of three such monomers. The Ad2 fiber is glycosylated and there are two N-acetylglucosamin molecules per fiber

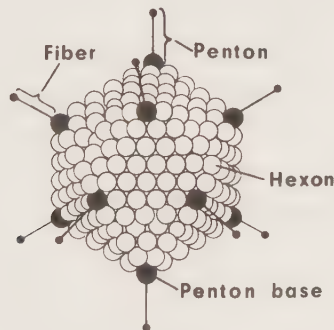


Figure 3. A schematic representation of an adenovirus particle, with indicated positions of the major structural components.
Redrawn from Norrby (82).

(43). This protein is linked to the penton base by noncovalent bonds to form the penton structure (6, 84, 90). The penton base from adenovirus types 2 and 5 is composed of a single polypeptide with an apparent molecular weight of 70,000 to 85,000. The molecular weight of the total penton capsomer has been estimated to be 485,000 to 505,000 for the type 5 penton (119) and between 370,000 and 400,000 for the type 2 penton (90).

Human adenoviruses are successfully propagated in various types of human cells. HeLa cells originate from a biopsy of a cervical carcinoma obtained in 1951 (40) and was the first human cell line to be established. Together with several other established human cell lines, the HeLa cells are most suitable for the analysis of the different stages in the productive cycle.

PREVIOUS AND CONCURRENT INVESTIGATIONS

The early interaction between adenoviruses and cells

Attachment of adenoviruses to cells has been studied in both the red blood cell system and the permissive cell system. The red blood cell may appear as a simple and convenient model for studies on virus attachment, because attached adenovirions do not penetrate the plasma membrane of these cells (8). The plasma membrane of red blood cells has furthermore been thoroughly studied (66, 106). However, in the permissive cell system, and under normal physiological conditions attached virions are rapidly internalized and uncoated, with a subsequent expression of the viral genes. With these things in mind, the results from virus-red blood cell interactions must be interpreted with caution, and extrapolations to analogous interactions in permissive cell systems should be made judiciously.

Cellular receptor sites for adenoviruses

Adenovirus type 7 (Ad 7) does not attach to ghosts of rhesus monkey red blood cells that are pretreated with different proteolytic enzymes

(79). This provides indirect evidence for the proteinaceous nature of the cellular receptor sites in this particular system. Even though neuraminidase-treatment does not inactivate the red blood cell receptor sites for Ad7, one may not exclude the possibility of a carbohydrate involvement. In fact, periodate treatment of such red cell ghosts results in a loss of virus binding, which implies a carbohydrate moiety in the receptor site structure. From monkey red blood cells a deoxycholate-solubilized receptor site protein with a molecular weight of 40,000 has been identified by chromatography on Sepharose 4B (79).

In the permissive system (e.g. HeLa and KB cells) proteases have also been used to destroy the Ad2 and Ad5 receptor binding activities (39, 92). Thus, in subtilisin-treated cells it takes about 6 hours to regenerate the binding capacity, and this is a process that is inhibited by puromycin and cycloheximide (92), which demonstrates the need for de novo protein synthesis. Removal of sialic acid residues on the cell surface, by treatment with neuraminidase, has no negative effect on the attachment of adenovirus type 2 (7). In contrast the attachment is stimulated, even though it is just the initial attachment rate that is affected (7, Svensson & Persson, unpublished results). Interestingly, periodate treatment of cells or the addition to cells of the lectin concanavalin A inhibit the adsorption of the fiber antigen to a certain extent (39). These results suggest that some carbohydrate moiety of the receptor site, as in the red blood cell system, still may be important for a proper attachment of virions. On the other hand, the attachment of Ad2 to tunicamycin-treated HeLa cells is stimulated (Svensson & Persson unpubl. res.). Tunicamycin specifically inhibits N-glycosylation (112), which is the predominant type of protein glycosylation in the cell (88, 122). The fact that cells treated with neuraminidase or grown in the presence of tunicamycin promoted virion attachment, might be a consequence of a reduction in the number of repulsive electrostatic charges normally exposed on the cell surface.

Attempts have been made to isolate the cellular receptor sites of KB cells for adenovirus types 2 and 5 (30, 71). Thus, a water-soluble cellular glycoprotein with an apparent molecular weight of 75,000 has been partially purified on immobilized fiber antigen (71). Furthermore, three different approaches of affinity chromatography have been compared

in a study by Hennache and Boulanger (30) for the isolation of a detergent-solubilized CRS for Ad2. The isolated polypeptides from KB cells, common for all three procedures, displayed molecular weights of 78,000, 42,000 and 34,000. These isolated membrane proteins were also shown to impair the virus attachment in competition experiments.

Adenovirus attachment to cells

As mentioned above, all human adenovirus serotypes agglutinate red blood cells from different mammalian species. Together with the intact virion, dimers and multimers of pentons and fibers are all capable of hemagglutination, whereas the corresponding monomer forms fail to do so (81, 85, 120, 121). This not only strongly suggests that the fiber antigen is the virus attachment protein, but also that the multivalency of this component is a prerequisite for the agglutination of the red blood cells.

In the permissive cell system it is also generally accepted that the fiber antigen is the VAP, since pretreatment of cells with this antigen inhibits subsequent virus attachment (92). The number of binding sites (i.e. CRU) on HeLa and KB cells for the Ad2 fiber has been estimated to be about 10^5 to 10^6 (92), while the reported value for adenovirus type 5 fiber on isolated KB cell plasma membranes is 90,000 (39). In the latter case, an association constant of around $1.7 \times 10^8 \text{ (M}^{-1}\text{)}$ was estimated from a Scatchard analysis of the binding data. The saturation level for binding of intact Ad2 virions on HeLa and KB cells is around 10^4 virus particles per cell, which is one to two orders of magnitude below the corresponding level for isolated fiber molecules (92).

A labilization of the adenovirus capsid occurs early in the virus infection, which renders the viral genome sensitive to treatment with deoxyribonuclease (55, 91, 109). As early as 20 minutes after the virus addition to cells, 80 % of the virus-DNA becomes accessible to enzyme degradation (55, 91). It has been suggested that the vertex regions of the virus particles are lost during the penetration of the plasma membrane (55, 109), but also contradictory ideas have been put forward favouring destabilization at a later stage (63, 74). The destabilization process may take place even when virus particles are incubated with

isolated plasma membranes (9), indicating that the process occurs in close connection to the cell surface.

The attachment of Ad2, both to red blood cells and to HeLa cells, is strongly temperature dependent (42, 91). The attachment rate is about seven times faster at 37 °C than at 0 or 3 °C (42, 91). The requirement for plasma membrane proteins to migrate laterally in the membrane at adenovirus attachment, is supported by different electron microscopic studies of infected KB cells (31, 32, 89). It was demonstrated that virus attachment at low temperatures induced rearrangement and clustering of intramembraneous particles, that disappeared upon warming the cells to 37°C (31). In addition, attachment of Ad2 was shown to induce redistribution of sterols in the plasma membrane (32). A likewise temperature dependent redistribution of Ad2 particles attached to HeLa cells was shown by Patterson and Russell (89). Inhibition of this latter virus-receptor site movement was revealed in the presence of sodium azide and cytochalasin B. These agents also blocked the subsequent internalization process.

THE PRESENT INVESTIGATION

Isolation and immunochemical identification of a cellular receptor protein for adenovirus type 2 (I, V)

HeLa cell plasma membranes were isolated in a two-phase polymer system consisting of polyethylene glycol and dextran (10, 44). From these membranes, proteins were solubilized at high salt concentration, with organic solvents, or with different detergents. To compare the release of receptor material by the different extraction procedures, the extracts were incubated together with radioactively labeled Ad2 particles, and the subsequent attachment of these virions to HeLa cells was followed. In a normalized assay, such attachment data were compared with attachment results of untreated Ad2. We found that 0.5 % Triton X-100 (TX100) was the most efficient agent in solubilizing so called receptor-active components, which to various degrees interfered with virion attachment.

Proteins of 0.5 % TX100-solubilized membranes sedimenting together with Ad2 particles in sucrose gradients were further isolated. A quantitative difference was discernible in the amounts of membrane proteins associated to virions in excess, and in the presence of different concentrations of TX100. At a concentration of 0.037 % TX100, 0.3 % of the solubilized proteins adsorbed to Ad2 virions. In sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analyses (65) of this protein fraction, a 40,000 molecular weight polypeptide was revealed.

In order to isolate larger quantities of solubilized membrane proteins with affinity for Ad2 particles, a two-step affinity chromatographic procedure was developed. First, proteins with affinity for wheat germ agglutinin (WGA) were isolated. These glycosylated proteins were subsequently separated on a column of glutaraldehyde-fixed Ad2 particles immobilized to Sepharose 4 B, via a 6-carbon spacer. The fraction of protein(s) with high affinity for the virions was eluted at low pH (i.e. pH=2.6), and corresponded only to about 1 % of the totally solubilized plasma membrane protein content. SDS-PAGE analysis of this material displayed two polypeptides, one predominant species with a molecular weight of 42,000 and a minor of 40,000. Paradoxically, this fraction of membrane proteins did not impair the viral attachment to cells in competition experiments. The reason for this might be that the low pH elution destroyed the native structure and naturally exposed determinants of the membrane protein(s).

An antiserum inhibiting the virus attachment was recovered after repeated immunizations in rabbits of isolated plasma membranes. This antiserum was characterized in crossed immunoelectrophoretic (CIE) analyses (54) of 1 % TX100-solubilized membrane proteins. A reference pattern of 20 immunoprecipitates was reproducibly obtained. In CIE with an intermediate gel (3, 110), containing immobilized fiber antigen, only one precipitate (designated I) in the reference pattern was diminished. This precipitate was not affected if the hexon antigen was used in the intermediate gel, nor was any of the other precipitates. On the other hand, WGA reduced the heights of about one third of all the precipitates of the reference pattern, including the one affected by the fiber. Direct elution (86) of this latter precipitate recovered from crossed immunoelectropherograms of iodinated membrane proteins, showed in

SDS-PAGE a polypeptide with the molecular weight of 39,000. In order to obtain an antiserum with higher relative specificity towards this membrane component, constituting precipitate I, this antigen-antibody complex was cut out directly from crossed immunoelectropherograms and injected into rabbits. In the CIE reference pattern precipitate I is closely surrounded by other precipitates, therefore one of the most dominating precipitates (designated II) in this region was also used as an immunogen. Thus, the recovered antiserum against the latter immunogen was initially meant as a "background"-antiserum. Curiously, it was this antiserum against precipitate II that exhibited the pronounced inhibitory effect on the viral attachment. CIE of membrane proteins with the recovered antisera, demonstrated that the anti-precipitate II antiserum precipitated the membrane component constituting precipitate I, whereas anti-precipitate I antiserum did not. Therefore it was concluded that antibodies against the glycosylated membrane protein, with the molecular weight of 39,000, also had the capacity to inhibit the viral attachment. This membrane component is probably identical with the protein isolated from immobilized Ad2 particles (see above), and furthermore is the component of the cellular receptor sites for Ad2 on HeLa cells.

The mechanism of adenovirus attachment (II, III & IV)

Different aspects of the temperature dependence of the Ad2 attachment to cells were studied under controlled conditions. The initial rate of the attachment was strongly affected by the temperature and was around 10 times faster at 37 °C than at 3°C. The attachment kinetics revealed an inflection point at around 20 °C, and above this temperature the increase of the rate was doubled. A corresponding inflection point was absent when Ad2 was attached to cells stabilized with 0.015 % glutaraldehyde. In multiplicity dependence experiments, the attachment was studied at equilibrium, where Ad2 virions were added to cells at different multiplicities of infection, ranging between 50 and 20,000. Scatchard plots (98) of the results revealed a positive cooperativity at 37 °C. From the same analyses the number of binding sites (CRS) for Ad2 on HeLa cells was estimated to about 5,000 and the association constant between the interacting components was determined to $1.5 \times 10^{10} (\text{M}^{-1})$ per site. The positive cooperativity of Ad2 binding was inhibited by low

temperatures (e.g. 3 °C) or by stabilization of cells with glutaraldehyde. The degree of cooperativity was quantified by means of Hill coefficients (N_H) obtained from Hill plots (34) of the binding data. The Hill coefficient at 37 °C was around 1.4, whereas it was just below unity at 3 °C or for attachment of Ad2 to glutaraldehyde-fixed cells. A value for the Hill coefficient below 1.0 indicates a negative cooperativity for the ligand-receptor interaction under study. The engagement of two types of receptor sites with different affinity for adenovirions was also revealed after computerized model fitting of the experimental data at 3°C. The virus binding to the high affinity sites displayed an association to cells with the same binding affinity as at 37 °C. On the other hand, the association of virions to the low affinity sites corresponds to a binding affinity in the range of that obtained for isolated fiber (see below). The temperature dependence of the cooperative binding was further analyzed at temperatures between 3 and 37 °C. The Hill coefficients increased progressively from 10 °C to reach a maximum level, which was maintained between 20 and 37 °C. Analogous multiplicity dependence experiments with purified fiber antigen, demonstrated weak positive cooperativity at 37 °C ($N_H = 1.1$), which was absent at 3°C ($N_H = 1.0$). The mean number of receptor units (CRU) for the fiber on HeLa cells was estimated to 90,000 with a mean association constant per site of $2.3 \times 10^8 \text{ (M}^{-1}\text{)}$, both at 3 and 37 °C. The effect of different reagents on the positive cooperative binding of Ad2 was studied at 37 °C. Dithiothreitol and dansylcadaverine markedly reduced the degree of positive cooperativity, while agents reducing the ATP content in the cell or interfering with the cytoskeletal elements, displayed no effect. Treatments of cells with EDTA or EGTA were also negative.

To directly visualize any possible movement of virus-receptor complexes in the membrane, rhodamine was conjugated to virions and the attachment of such virions was followed by fluorescence microscopy. At 37 °C capping of virions was observed on 15 % of the scored cells. The capping phenomenon was inhibited by low temperatures, prefixation of the cells with glutaraldehyde and by reagents such as sodium azide, 2-deoxyglucose and cytochalasin B.

The destabilization of Ad2 virions occurring early in infection, which renders the viral genome sensitive to DNase-treatment (55, 91, 109), was also shown to be temperature dependent. An Arrhenius plot (2) of the destabilization process at different temperatures, revealed an inflection point at around 16 °C, with calculated activation energies of 88 kJ per mol above and 331 kJ per mol below this temperature. These values are in correspondence to reports for membrane processes above and below lipid phase transitions (47) and for established systems of endocytosis (64). Interestingly, the destabilization of virus particles was shown to take place even though the internalization process was inhibited by sodium azide. We therefore analyzed the capacity of isolated plasma membranes or cell homogenates to mediate this process. The efforts to destabilize adenovirions in vitro turned out negative, thus indicating the need for intact and metabolizing cells to provide the proper microenvironment for this event to occur. Addition of dithiothreitol or dansylcadaverine markedly impaired the DNase-sensitivity of the viral-DNA, which is notable in the case of dithiothreitol treatment, since internalization of virions was almost unaffected in the presence of this reagent. Examination in the electron microscope of cells 20 minutes after the addition of Ad2 and in the presence of dithiothreitol, revealed that almost 70 % of the internalized virus particles were located in large, noncoated cytoplasmic vesicles. This frequency should be compared with the around 7 % for a control series of untreated cells. The chelating agents EDTA and EGTA inhibited both the destabilization process and the internalization of virions.

In summary, the temperature dependence of the different events connected to attachment of adenovirions seem to be a reflection of the lateral diffusability of the cellular receptor units in the membrane. From the results in this investigation we suggest the following sequence of events during the Ad2 attachment to cells:

- 1) initial attachment of virions to one or two cellular receptor units occurs with a binding association corresponding to that of isolated fiber protein;
- 2) recruitment to the initial virus-receptor unit complex of cellular receptor units, in the plane of the plasma membrane, is required to form CRSs constituting a multivalent interaction with the virus. The association between the interacting components at this stage is 60 times stronger than for the initial binding;

- 3) the migration of virus-cellular receptor-site complexes yielding a capping phenomenon on a minor fraction of the infected cells, is most likely a process not of vital importance for the productive infection as evidenced by the sensitivity of the capping to cytochalasin B. This drug has been shown not significantly to impair the other studied early events of the virus infection.

These suggestions nicely corroborate the hypothetical scheme of events for early interactions between animal viruses and host cells, as proposed by Lonberg-Holm and Philipson (62). The lateral diffusion of the cellular receptor units as described under 2) provides the basis for the positive cooperative binding of Ad2, but also for the factual destabilization of attached virions, occurring already on the outside of the plasma membrane.

CONCLUDING REMARKS

The results in this investigation suggest that the major component of the cellular receptor sites for adenovirus type 2 on HeLa cells is a glycosylated protein with a polypeptide molecular weight of around 40,000. In an analogous study, Hennache and Boulanger studied proteins from KB cell plasma membranes with affinity for Ad2 particles (30). They isolated a pertinent protein fraction consisting of three polypeptides with the apparent molecular weights of 78,000, 42,000, and 34,000. These authors suggested that the isolated 42,000 polypeptide might be identical with actin, the major component of the cytoplasmic microfilament (50). The polypeptide identified and isolated in the present study, was shown to have an electrophoretic mobility in agarose gels different from that of actin (Persson & Svensson, unpubl. res.). Since two groups, working independently with two different human cell lines, have isolated a CRS-polypeptide with similar molecular weight, it would be worthwhile to study the Ad2 attachment to KB cells, pretreated with the recovered antiprecipitate II antiserum described above. Such a study will provide evidence for a possible sharing among HeLa and KB cells of components of the CRS for Ad2. Since the KB cells are derived from a human oral epidermoid carcinoma and the natural infections by adenoviruses cause mainly respiratory diseases, such a study would cast some light on the

biological relevance of CRSs on human cells of different origin. In order to obtain more detailed information about the CRSs for Ad2, large amounts of pure protein(s), isolated under native conditions, will be needed. Then, both a more complete biochemical characterization, together with a biological ditto, may be realistic. One way to attack the isolation problem may be to produce monoclonal antibodies (52) against either total plasma membrane proteins or preferably against protein fractions of "receptor-active" material - and to use the relevant antibodies in affinity chromatographic procedures. To screen for monoclonal antibodies against receptor site component(s), the assay for inhibition of virus attachment could be employed. With the appropriate antibodies at hand it would also be possible to study the epitopes of the CRS that bind the VAP.

Herein are also presented suggestive evidence for the requirement of diffusion of cellular receptor units in the plasma membrane for the virus attachment to cells. As a consequence of the CRU migration, the Ad2 binding exhibits positive cooperativity, and - with an additional dependence of bivalent cations - the attached virions become destabilized. To directly measure the migrational behaviour of CRUs in the plasma membrane, one has to use fluorescent probes such as the actual viruses or CRUs in connection with the technique of fluorescence recovery after photobleaching (56, 93). The dependence of bivalent cations, for the destabilization process may indicate the involvement of an enzymatic reaction, e.g. a protein kinase activity of the CRSs. Such an activity has been stated for the receptors for insulin and epidermal growth factor (107).

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Virus-Receptor Interaction in the Adenovirus System

I. Identification of Virion Attachment Proteins of the HeLa Cell Plasma Membrane

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Plasma membranes from HeLa cells were isolated in a two-phase polymer system. To compare the efficiency of attachment protein extraction, a normalized assay for the assessment of adenovirus type 2 (Ad2) receptor-active components interfering with the attachment of Ad2 to HeLa cells was developed. An optimized detergent extraction procedure, 0.5% Triton X-100, was used, and solubilized membrane proteins were radioisotope labeled *in vitro*. Proteins with affinity for Ad2 virions were quantified and identified in a sucrose gradient sedimentation assay and by affinity chromatography with cross-linked Ad2 virions immobilized to AH-Sepharose 4B. From virions recovered in the sucrose gradient system, one major membrane component of high affinity was identified with a polypeptide molecular weight of around 40,000. Glycosylated proteins isolated by wheat germ lectin chromatography with high affinity for immobilized virus particles were isolated, and two major components with apparent molecular weights of 40,000 and 42,000 were identified. We suggest that a glycosylated protein with high affinity for Ad2 virions and a polypeptide molecular weight of 40,000 to 42,000 is one component of the Ad2 attachment site on HeLa cells.

All virus infections start with the viruses recognizing components on the surface of the cell that is about to be infected. The extracellular interactions between animal viruses and cells have been extensively reviewed and described by Lonberg-Holm and Philipson (27). More recently, Kohn has elaborated on a selected number of viruses interacting with cellular membranes (24).

The cellular surface components of interest for the infecting virions have usually been referred to as viral receptors. A more appropriate term, virus attachment proteins, has been suggested (32); this term implies that the cells do not synthesize membrane components for the *a priori* function of being the structures responsible for reception of future viral infections.

In the adenovirus system, the virion part of the virus-cell-recognizing system is the well-characterized fiber antigen (10, 34, 37) located at the 12 vertices of the virion (38). Previous studies also indicate that plasma membrane components that are protein in nature, and possibly glycosylated, are important in the initial steps of virus-cell interaction (31, 34). Attempts have been made to isolate and characterize the components of the attachment site for adenovirus type 2 (Ad2) on KB cells, using highly purified fiber and penton structures. Hennache and Boulanger (18) identified three major Ad2-fiber-rec-

ognizing polypeptides with apparent molecular weights of 78,000, 42,000, and 34,000.

With an assay designed to measure and normalize adenovirus attachment protein activity of HeLa cell plasma membrane extracts, we tried to optimize the conditions for preparation of virus recognition proteins. Throughout efforts to identify and isolate these components, *i.e.*, by sucrose gradient sedimentation analyses and affinity chromatography techniques, complete virus particles were used to mimic the complex situation *in vivo*.

MATERIALS AND METHODS

Cells. HeLa cells were maintained in suspension cultures at cell densities of 2×10^5 to 6×10^5 cells/ml in Eagle minimal essential medium (EMEM) supplemented with 8% fetal calf serum and gentamicin ($5 \mu\text{g/ml}$).

Infections and virus purification. HeLa cells grown in spinner cultures were synchronously infected with Ad2 at a multiplicity of 2,000 particles per cell as previously described (13). For radioisotope labeling, virus was propagated in the same way. [^3H]thymidine (56 Ci/mmol ; $25 \mu\text{Ci/ml}$) was added 10 h postinfection and maintained until harvest of cells at around 40 h postinfection. Virus was purified and stored as described elsewhere (12).

Plasma membrane isolation. HeLa cell plasma membranes were isolated from batches of 5×10^8 cells in a discontinuous sucrose gradient system as described by Bosmann *et al.* (5), modified with a cushion

of 60% (wt/vol) sucrose applied at the bottom of the centrifuge tubes. Five fractions were collected from each tube and denoted as follows: fraction I, the cushion of 60% sucrose; fraction II, the density region corresponding to the interphase between the sucrose concentrations of 45 and 35%, including the entire portion of 45% sucrose; fraction III, the interphase region between the sucrose concentrations of 35 and 25%; fraction IV, the interphase region between concentrations of 25 and 20%; and fraction V, the remaining uppermost portion.

Plasma membranes were also prepared in a two-phase polymer system consisting of polyethylene glycol 6000 and dextran T-500 as described by Brunette and Till (7) and modified by Jay et al. (22). Batches up to 2×10^9 cells were processed, and 5×10^8 cells per 120 ml of mixed-phase system were used. At the end of the separation, the interphase was collected and diluted five times in 50 mM Tris-hydrochloride buffer, pH 7.3. The membranes were sedimented at $4,500 \times g$ for 15 min at 4°C , washed once, and resuspended in the buffer above to yield a protein concentration of 4 to 8 mg/ml.

Enzyme marker assays. 5'-Nucleotidase was assayed by the method of Heppel and Hilmoe (19) as modified by Johnsen et al. (23). Na^+ , K^+ -ATPase was determined by the method of Wallach and Kamat (39). Phosphate determinations were in both cases done according to the method of Chen et al. (9). Reduced nicotinamide adenine dinucleotide phosphate cytochrome *c* reductase was assayed by the method of Hrycay and O'Brien (20). Protein was determined by the method of Hartree (16), with bovine serum albumin (BSA) as the standard.

Solubilization of membrane proteins. All solubilization experiments were carried out on plasma membranes isolated by the two-phase method.

(i) High-salt procedure. Solid KCl (36) was added to a final concentration of 3 M; the samples were then shaken until the salt dissolved and further incubated for 16 to 20 h at 8°C . The samples were subsequently centrifuged at $100,000 \times g$ for 1 h at 4°C , and the supernatants were dialyzed against 50 mM Tris-hydrochloride buffer, pH 7.3.

(ii) Organic solvent procedures. Butanol (29), pentanol (40), and pyridine (3) were separately applied as extracting agents, each at a final concentration of 10% (vol/vol). After incubation for 1 h at 8°C with shaking every 15 min, the samples were centrifuged and dialyzed as described for the high-salt procedure.

(iii) Detergent procedures. Various concentrations of the following detergents were used: Triton X-100 [polyoxyethylene(9,10)-*p*-*t*-octyl-phenol], Tween 20 (polyoxyethylene sorbitan monolaurate), and sodium deoxycholate (DOC) (17). After detergent treatment, the samples were incubated for 1 h at 8°C with shaking every 15 min and finally centrifuged as above. The dialysis step was omitted.

In vitro labeling of proteins. Triton X-100-solubilized proteins were labeled for 2 h at 26°C by reductive alkylation, using sodium cyanoborohydride and [^3H]formaldehyde as described by Dottavio-Martin and Ravel (11). The reaction mixtures were passed through PD-10 columns with Sephadex G-25M equilibrated and eluted with 40 mM sodium phosphate buffer, pH 7.0, containing 0.037% Triton X-100. The

labeled proteins were recovered in the void volume and monitored by assessment of trichloroacetic acid-precipitable radioactivity. The specific radioactivity of labeled proteins ranged from 2.7×10^7 to 3.0×10^7 cpm per mg of protein.

Labeling of membrane proteins with the Bolton-Hunter reagent was performed essentially as described by the manufacturer. After labeling, the samples were separated from unincorporated isotope by passing through a PD-10 column with 0.15 M NaCl-0.037% Triton X-100 in 0.015 M sodium phosphate buffer, pH 7.3, as the eluant. Labeled material was detected by radioactivity measurements without prior precipitation. Labeling efficiencies were in the range of 90%, and the specific activities of labeled material were about 9×10^8 cpm/mg of protein.

Assay of receptor-active material. The principle of the assay was based on the assumption that solubilized plasma membrane proteins compete with viruses during the course of virus-cell interaction and thus inhibit virus attachment to cells.

(i) Incubation 1. Five microliters of highly purified [^3H]thymidine-labeled Ad2 virions (8.5×10^9 particles; 69,400 cpm) was mixed with 120 μl of double-distilled water (for the control series) or with 120 μl of protein sample (for the sample series) and 125 μl of 2 $\times$ EMEM designed for suspension cultures without serum, which was omitted in all subsequent steps. To ensure proper solubility of all reagents, Triton X-100 was added at a final concentration of 0.037% to all incubation mixtures. When extracts containing Tween 20 or DOC were analyzed, the final concentrations of these detergents also were adjusted to 0.037%. This concentration of these detergents has previously been shown not to affect virus attachment to HeLa cells. The mixtures were incubated for 1 h at 37°C on a shaking water bath and then centrifuged at $140 \times g$ for 5 min at room temperature. The supernatants were transferred to round-bottom plastic tubes, and 2.5×10^7 previously EMEM-washed HeLa cells were added in a volume of 250 μl . This gave a final concentration of 5×10^7 cells/ml and a multiplicity of infection of about 340 particles/cell.

(ii) Incubation 2. The cell-containing mixtures were incubated for 1 h at 37°C as above, and samples were withdrawn at different intervals, diluted 10 times in EMEM, and centrifuged at $140 \times g$ for 3 min. The radioactivity in the supernatants and the pellets was measured and used for calculating the degree of virus attachment. Three-point attachment curves were always constructed for the samples analyzed.

(iii) Calculations. The percentages of radioactivity remaining in the supernatants after 60 min of attachment were used to calculate the relative inhibition of virus attachment. This procedure was believed to counteract any possible variations in the status of the cells. Calculations were performed according to the formula:

relative inhibition

$$= \frac{\begin{array}{l} \% \text{ cpm in supernatant (sample)} \\ - \% \text{ cpm in supernatant (control)} \end{array}}{\begin{array}{l} \% \text{ cpm in supernatant} \\ \text{(maximum inhibition)} \\ - \% \text{ cpm in supernatant (control)} \end{array}} \times 100$$

where percentage of counts per minute in supernatant (sample) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the presence of presumptive receptor material; percentage of counts per minute in supernatant (control) is the radioactivity remaining in the supernatant after 60 min of virus-cell interaction in the absence of interfering material; and percentage of counts per minute in supernatant (maximum inhibition) is the radioactivity remaining in the supernatant at maximum inhibition of attachment in the presence of, for example, detergent-extracted plasma membrane proteins. The highest measured inhibition was 95% after 60 min of virus-cell interaction. The relative inhibition revealed by different quantities of Triton X-100 (0.5%)-extracted proteins was determined, and a dose-response curve was constructed (see Fig. 3 in Results). We assumed that dose-response curves of similar shape would be obtained with proteins of other extraction procedures. This would enable us to compare the efficiency of virus attachment inhibition of other protein extracts. The degree of inhibition of all extracts was normalized to a 50% relative inhibition of attachment in the Triton X-100 system, using 0.5% detergent at extraction.

Rate zonal sedimentation analysis of virus-attached plasma membrane proteins. Highly purified Ad2 virions were mixed with solubilized plasma membrane proteins radioactively labeled *in vitro*. The amount of virus and protein used corresponded to multiplicities of infection between 6,000 and 8,000 particles per cell equivalent based on calculations of extraction efficiencies and original cell quantities used for plasma membrane preparations. The samples were incubated in the presence of 0.037% Triton X-100, 0.5% Triton X-100, 0.2 M NaCl, 1% BSA, or 3% BSA for 1 h at 37°C with shaking. Except for the incubation with 0.5% Triton X-100, all incubation mixtures were supplemented with Triton X-100 to give a final concentration of 0.037%. The mixtures were subsequently layered onto linear sucrose gradients consisting of 2 × 1.6 ml of 20 to 40% (wt/vol) sucrose in 50 mM Tris-hydrochloride buffer, pH 7.5, and containing the same amount of detergent, salt, or BSA as the pertinent incubation mixture. The gradients were underlaid with 0.2 ml of 60% (wt/vol) sucrose in the same buffer system and centrifuged at 100,000 × *g* for 40 min at 6°C. After fractionation into 25 fractions by puncturing from the bottom, radioactivity was monitored by counting 5-μl portions. Fractions of interest were pooled, dialyzed against double-distilled water, freeze-dried, and analyzed electrophoretically on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels.

Chemical cross-linking of Ad2 virions. Glutaraldehyde was used to stabilize Ad2 by cross-linking in the presence of 0.1 M sodium phosphate buffer, pH 7.4, in a procedure described by Avrameas and Ternyck (2) with some modifications. The concentrations of glutaraldehyde and virions were determined so as to maintain the virus particles in a true soluble state as well as provide the highest coupling efficiency of virions to AH-Sepharose 4B. In the procedure chosen, glutaraldehyde (25% solution) was added to a 5 optical density units solution of Ad2 in sodium phosphate buffer to make the concentrations of glutaraldehyde and sodium phosphate 5% and 0.1 M, respectively.

The total 3-ml volume of the mixture was incubated for 24 h at 8°C, whereafter it was freed of glutaraldehyde by filtering through a PD-10 column with Sephadex G-25M in 0.1 M sodium phosphate buffer, pH 7.4. Fractions were pooled after measuring the absorption at 260 nm. The stabilized Ad2 virions were further immobilized and used as an affinity adsorbent (see below).

Fractionation of material with Ad2 virion affinity from the HeLa cell plasma membrane. (i) Wheat germ lectin-Sepharose 6MB chromatography of solubilized membrane proteins. A column with 5 ml of wheat germ lectin-Sepharose 6MB was equilibrated with WGA buffer (0.2 M NaCl and 0.037% Triton X-100 in 0.05 M sodium phosphate buffer, pH 7.0). [³H]formaldehyde-labeled, Triton X-100 (0.5%)-solubilized membrane proteins were applied to the column after dialysis against WGA buffer for 18 to 20 h at 4°C. Fractions of 0.5 ml were collected at a flow rate of 3.4 ml/h. After the column was washed with WGA buffer, the retained proteins were eluted with *N*-acetyl-D-glucosamine (100 mg/ml) in WGA buffer. The elution profile was followed by direct counting of 10-μl portions from each fraction. The peak fractions of interest were pooled and dialyzed against WGA buffer for 20 h at 4°C.

(ii) Affinity chromatography on an immobilized adenovirion matrix. Highly purified Ad2 virions were stabilized by cross-linking as described above and incubated with carefully washed AH-Sepharose 4B (up to 2.5 optical density units of Ad2 per ml of gel) for 24 h at 4°C in a rolling state. Ethanolamine was added (1 M, final concentration) to block any residual aldehyde groups, and the mixture was incubated for another 12 to 15 h at 4°C. After the second incubation, the gel was washed extensively with WGA buffer and was then ready to use. By using radioactively labeled Ad2 virions, it was established that the coupling efficiency to AH-Sepharose 4B was 90 to 95%. Moreover, when coupled viruses were treated with buffer solutions at low pH (0.2 M glycine-hydrochloride, pH 2.2), at high pH (0.2 M glycine-NaOH, pH 12.0), at high ionic strength (5 M MgCl₂ in 50 mM Tris-hydrochloride, pH 7.4), and containing detergent (1% Triton X-100 in 50 mM Tris-hydrochloride, pH 7.4), no loss of virus was detected. Therefore, it was assumed that the viruses were covalently linked to the matrix.

Immobilized virions on an Ad2-AH-Sepharose 4B column were able to adsorb antifiber antibodies, indicating the presence of immunologically intact and accessible fibers of the virions. This immunological procedure was used to monitor the stability of the columns used throughout these investigations.

Unretained and retained fractions from the lectin chromatography step (see above) were analyzed on the Ad2-AH-Sepharose 4B (1.2 optical density units of Ad2 per ml of gel), equilibrated with WGA buffer. The pertinent fractions were applied to the column, and 3.2-ml fractions were collected at a flow rate of 3.4 ml/h. After washing of the column with WGA buffer, proteins of low virus affinity were eluted with WGA buffer containing 0.5 M NaCl. Proteins with high affinity for Ad2 virions were subsequently eluted with 0.1 M NaCl-0.037% Triton X-100 in a 0.1 M glycine-

hydrochloride buffer, pH 2.6. Fractions obtained from elution at low pH were immediately neutralized with 1 M sodium phosphate buffer, pH 7.2. The elution profiles were monitored by direct radioactivity measurements of 50- μ l portions withdrawn from each fraction. Fractions constituting the peaks of interest were pooled, dialyzed against double-distilled water, and freeze-dried.

SDS-PAGE. The SDS-polyacrylamide gel electrophoresis (PAGE) analyses were performed on gel slabs (1.5 by 70 by 70 mm) with 13% acrylamide and 0.35% bisacrylamide essentially as described by Maizel (30). Fluorography was done according to Bonner and Laskey (4).

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were done in a cocktail of toluene-methanol (1:1) with 0.4% Omnifluor. Assessment of trichloroacetic acid-precipitable radioactivity was done on filter paper disks (Munktell, Grycksbo, Sweden, OOH; 21-mm diameter). Filters with added and dried samples were submerged into 10% ice-cold trichloroacetic acid for at least 30 min and then washed twice in 5% cold trichloroacetic acid and twice in 70% cold ethanol. The dried filters were counted in toluene with 0.4% Omnifluor. The ^{125}I radioactivity was measured in the tritium channel with a 60% efficiency. A Nuclear-Chicago Mark II liquid scintillation counter was used to determine radioactivity.

Chemicals and radioisotopes. EMEM, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. [^3H]formaldehyde (0.1 Ci/mmol), [^3H]thymidine (56 Ci/mmol), Bolton-Hunter reagent (^{125}I ; 2,000 Ci/mmol), and Omnifluor were purchased from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. *N*-acetyl-D-glucosamine, Triton X-100 (scintillation grade), DOC, and polyethylene glycol 6000 were from BDH Chemicals Ltd., Poole, England; Tween 20 and sodium cyanoborohydride were from Sigma Chemical

Co., St. Louis, Mo. Wheat germ lectin-Sepharose 6MB, AH-Sepharose 4B, dextran T-500, and PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. Kodak XG-14 X-ray films were used in the autoradiography and fluorography analyses and were developed in Kodak D-19, both products from Eastman Kodak Co., Rochester, N.Y. Glutaraldehyde (25% for electron microscopy) was from E. Merck AG, Darmstadt, West Germany.

RESULTS

Comparison of procedures for plasma membrane isolation. Two methods were studied for plasma membrane isolation as described in Materials and Methods. The recoveries of protein and distributions of marker enzymes are shown in Table 1. Based on the greater recovery of protein, the higher enrichment of 5'-nucleotidase activity, the facts that the two-phase separation procedure is faster, less laborious, and more suitable in a preparative scale, this technique was chosen for further plasma membrane preparations (6). The purity of the plasma membranes prepared by this method was controlled in a phase-contrast microscope and electron microscope, and no contamination of intact cells, nuclei, mitochondria, or granular elements was observed (data not shown).

Solubilization of plasma membrane proteins. The efficiencies of membrane protein release by various functionally distinct solubilization techniques were compared (Table 2). Some extractions were performed in such a way that one solubilization technique was followed by another in order to release material from the nonextractable remainder of the previous pro-

TABLE 1. Comparison of methods for plasma membrane isolation^a

Method and sample	Protein recovery (%)	5'-Nucleotidase		Na ⁺ ,K ⁺ -ATPase		NADPH cytochrome c reductase		Recovery (%)
		Sp act (μmol of PO_4^{3-} /h and mg)	Relative sp act	Sp act (μmol of PO_4^{3-} /h and mg)	Relative sp act	Sp act (μmol /h and mg)	Relative sp act	
Bosmann et al. (5)				ND		ND		
Homogenate	100	1.7 $\pm$ 1.1 (3)						
Supernatant	50.4 $\pm$ 36.2 (3)	1.2 $\pm$ 0.9 (3)	0.9 $\pm$ 0.4 (3)					
Fraction II ^b	1.5 $\pm$ 0.17 (3)	4.7 $\pm$ 1.8 (3)	3.6 $\pm$ 1.9 (3)					
Brunette and Till (7)								
Homogenate	100	1.4 $\pm$ 0.8 (6)		0.29 $\pm$ (5)		0.49 $\pm$ 0.09 (4)		
Supernatant	62.5 $\pm$ 6.0 (13)	1.4 $\pm$ 0.4 (3)	0.8 $\pm$ 0.2 (3)	ND		0.56 $\pm$ 0.18 (4)	1.17 $\pm$ 0.37 (4)	106 $\pm$ 23.6
Interphase ^c	3.9 $\pm$ 0.9 (23)	9.3 $\pm$ 4.9 (6)	7.3 $\pm$ 2.8 (6)	2.10 $\pm$ 1.1 (5)	10.5 $\pm$ 8.4 (5)	0.85 $\pm$ 0.27 (4)	1.72 $\pm$ 0.28 (4)	3.7 $\pm$ 1.8

^a Values are expressed as mean $\pm$ standard deviation; number of experiments is given in parentheses. NADPH, Reduced nicotinamide adenine dinucleotide phosphate; ND, not determined.

^b Described in Materials and Methods.

In the two-phase separation system, the plasma membranes are enriched in the interphase.

TABLE 2. *Receptor activity (RI) of solubilized plasma membrane proteins recovered by different procedures^a*

Extraction procedure	Protein recovery (%)	Amt of protein needed for 50% RI (μ g)	Amt of protein needed for 50% RI normalized to the 0.5% Triton X-100 extraction system (μ g)
3 M KCl	17.6 $\pm$ 3.0 (4)	150 (1)	3.1
3 M KCl-10% pyridine ^b	4.5 $\pm$ 0.8 (2)	ND	
3 M KCl-0.5% Triton X-100 ^b	46.0 (1)	84 (1)	1.8
10% <i>n</i> -Butanol	12.0 (1)	ND	
10% <i>n</i> -Pentanol	17.7 (1)	ND	
10% Pyridine	20.8 $\pm$ 4.7 (5)	74 $\pm$ 17.6 (2)	1.5 $\pm$ 0.4
10% Pyridine-10% <i>n</i> -pentanol ^b	0 (1)	ND	
1.0% DOC	69.7 (1)	59 (1)	1.2
0.1 % Triton X-100	25.2 (1)	NE (1)	
0.5 % Triton X-100	37.6 $\pm$ 4.9 (14)	48 $\pm$ 13.2 (25)	1.0 $\pm$ 0.2
1.0% Triton X-100	43.9 $\pm$ 6.9 (3)	52 $\pm$ 7.1 (2)	1.1 $\pm$ 0.2
0.5% Triton X-100 - PD-10 ^c		71 $\pm$ 26.9 (2)	1.5 $\pm$ 0.6
0.5 % Tween 20	18.8 $\pm$ 6.3 (3)	NE (10)	
1.0% Tween 20	30.6 $\pm$ 6.8 (4)	NE (5)	
Total membranes		86 $\pm$ 19.6 (11)	1.8 $\pm$ 0.4

^a The relative inhibition of virus attachment was calculated as described in Materials and Methods. Where applicable, values are followed by standard deviation, with the number of experiments given in parentheses. ND, Not determined; NE, no effect (highest amount of protein analyzed was 80 μ g).

^b A second extraction of the nonextractable remainder of the first was done as described in Materials and Methods.

^c Before analysis in the attachment assay, the protein extracts were deprived of free detergent by passage through a PD-10 column followed by extensive dialysis.

cedure. It is evident that the detergent methods released the highest quantities of membrane protein. The high-salt and the organic solvent procedures were one-third to one-half as efficient.

When we compared the total polypeptide contents of the high-salt and the detergent extracts in SDS-polyacrylamide slab gels, we found that the solubilization by detergents enriched a number of proteins compared with the starting material (Fig. 1). Qualitatively, all extracts displayed similar polypeptide patterns. The electropherogram also supported the general concept about the versatility of proteins constituting the protein moiety of the HeLa cell plasma membrane (8).

Identification of receptor-active material. Solubilized plasma membrane proteins obtained from the high-salt, organic solvent, and detergent extraction procedures were analyzed

for the presence of material interfering with the process of adenovirus attachment to HeLa cells. A prerequisite for this analysis was the development of an assay for receptor-active material and its subsequent normalization (see Materials and Methods). Initially, the kinetics of adenovirus attachment to HeLa cells was studied (Fig. 2). After approximately 40 min of virus-cell interaction, maximum attachment was attained. The increased values for the standard deviation of later measuring points could have resulted from physical stress on the cells after this time of incubation. The tendency of the function to decrease at later time points could have been due to the fact that virions at this stage of incubation had entered the interior of the cells (26) and thus avoided detection, since the samples were assayed for radioactivity directly and not hydrolyzed before analysis. The basis for the normalized relative inhibition calculations was the dose-response curve (Fig. 3). Compared with total membrane content, a significant amount of receptor-active material (i.e., material interfering with virus attachment) was present in the 0.5% Triton X-100 extracts. Resuspension of the nonextractable remainder provided a sample with low ability to inhibit virus attachment, and although extreme quantities were used, only a maximum relative inhibition of around 25% was achieved. Table 2 shows the efficiencies of various other plasma membrane protein extracts in the inhibition assay. The Triton X-100 procedure using 0.5% detergent gave a 50% relative inhibition of virus attachment, using the lowest amount of extracted protein. Extraction with this concentration of detergent also made it possible to perform complete dose-response analyses without removing the detergent (see Materials and Methods). To simplify the comparison of Triton X-100 extracts with other extracts, all estimates were normalized to the Triton X-100 (0.5%) system (Table 2). Although the DOC procedure gave good production of receptor-active material, it was not considered for further studies because it could strongly interact with proteins irreversibly. The 3 M KCl extraction method produced an extract with poor inhibitory effect. We tried to optimize the Triton X-100 extraction procedure by first removing membrane components of low interfering ability with 3 M KCl and thereafter apply the Triton X-100 system to the nonsolubilized remainder. This sequence of extractions yielded an extract with only half the efficiency of the standard Triton X-100 (0.5%) procedure (Table 2).

Identification of polypeptides attaching to adenovirions. Since the Triton X-100 (0.5%) method produced an extract with the highest inhibitory effect on virus attachment to HeLa

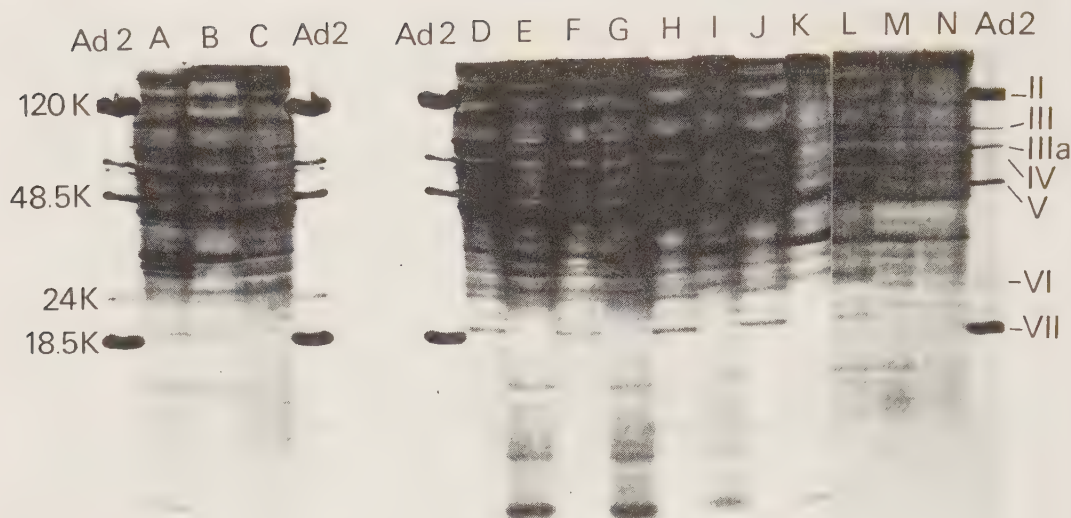


FIG. 1. Qualitative comparison in a stained SDS-polyacrylamide slab gel electropherogram of solubilized HeLa cell plasma membrane proteins. Each sample well was loaded with approximately the same amount of protein (75 μ g), and the gels were electrophoresed for a total time of 150 min, starting at 80 V for the first 20-min period and then increasing to 100 V. The contents of the lanes are as follows: (Ad2) marker Ad2 virions with indicated positions and molecular weights of major structural polypeptides as described by Anderson *et al.* (1); (A) 3 M KCl extract; (B) 0.5% Triton X-100 extract of the nonextractable remainder after a 3 M KCl extraction; (C) nonextractable remainder after consecutive extractions with 3 M KCl and 0.5% Triton X-100; (D) 0.5% Triton X-100 extract; (E) nonextractable remainder after a 0.5% Triton X-100 extraction; (F) 1% Triton X-100 extract; (G) nonextractable remainder after a 1% Triton X-100 extraction; (H) 0.5% Tween 20 extract; (I) nonextractable remainder after a 0.5% Tween 20 extraction; (J) 1% Tween 20 extract; (K) nonextractable remainder after a 1% Tween 20 extraction; (L) 1% DOC extract; (M) nonextractable remainder after a 1% DOC extraction; (N) total plasma membranes used for the various extractions. All extractions were performed as described in Materials and Methods except that the sample of lane M was recovered after centrifugation at $3,000 \times g$ for 30 min.

cells, this technique was chosen for further investigations. Solubilized and *in vitro* 125 I-labeled plasma membrane proteins were incubated with highly purified Ad2 virions as described in Materials and Methods. The incubated mixtures were subsequently layered onto sucrose gradients and sedimented as described. Reproducibly about 0.3% of the added radioactivity cosedimented with the virus particles in the presence of 0.037% Triton X-100 (Fig. 4). If the detergent concentration of the incubation mixture and gradient was increased to 0.5% Triton X-100, about 0.2% of the total radioactivity still remained associated with the virus. An additional increase of the detergent concentration up to 1% did not further reduce the amount of material attached to virions. In some instances, a low degree of virion and protein aggregation was discerned (0.06% of the total radioactivity) in the presence of both 0.037 and 0.5% Triton X-100. However, the majority of sedimenting labeled protein was always located in the region of the virus control. When 1 or 3% BSA was added to the incubation mixtures and gradients, we observed no mem-

brane proteins specifically cosedimenting with virions. When this large amount of protein was added (50 to 150 times more BSA per virus particle than membrane protein per virus particle), virus and membrane protein seemed to aggregate nonspecifically. Approximately 2.4% of the extracted membrane proteins were aggregated throughout the entire gradient when 3% BSA was used, compared with about 0.7% with 1% BSA.

Fractions from the sucrose gradient analyses of protein cosedimenting with virions were pooled as indicated in Fig. 4 and subjected to SDS-PAGE analysis as described in Materials and Methods. About 13 polypeptide bands were found associated with virions in the presence of 0.037% Triton X-100 (Fig. 5A). One of the predominant polypeptides had a molecular weight of approximately 40,000. The addition of 0.2 M NaCl had no effect on the qualitative pattern of polypeptides associated with the virus particles (data not shown), although the amount of proteins associated with virions was reduced to about 0.1%. If the detergent concentration was

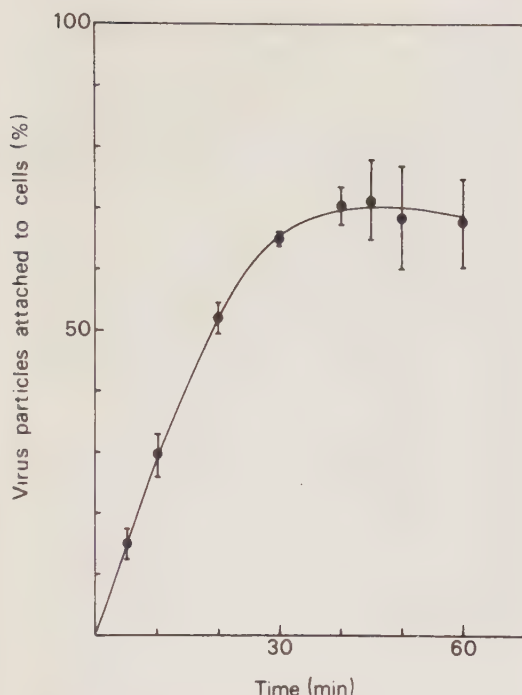


FIG. 2. Kinetics of adenovirus attachment to HeLa cells. Preincubated virus particles were mixed with HeLa cells, and samples were withdrawn at intervals after incubation began. The amount of radioisotope-labeled virus particles attached to cells was measured as described for the control series in Materials and Methods. Six separate experiments were performed, and the standard deviation is indicated for each measuring point.

increased to 0.5%, the 40,000-dalton polypeptide was the predominant species associated with virions, together with four faintly observable polypeptides (Fig. 5B). This 40,000-dalton polypeptide was probably identical to the protein species adsorbed with the highest affinity to the Ad2-AH-Sepharose 4B matrix (see below).

Lectin chromatography of solubilized plasma membrane proteins. Triton X-100 (0.5%)-solubilized and [3 H]formaldehyde-labeled membrane proteins were fractionated on wheat germ lectin-Sepharose 6MB as described in Materials and Methods. Radioactivity measurements reproducibly established that 5% of the total amount of protein material applied was retained by the column (pool B, Fig. 6).

Immobilized Ad2 as an adsorbent. Because adenovirus particles are fragile (25, 35), they must be stabilized before being immobilized and used as an adsorbent. Proteins of the virus particles were therefore cross-linked with glutaraldehyde and immobilized as described in Materials and Methods. These procedures pre-

served the ability of virions to interact with antifiber immunoglobulin G. Virions immobilized with no spacer reacted to a limited extent with antifiber antibodies, whereas the introduction of a six-carbon spacer produced a functional adsorbent (not shown).

Unretained and retained fractions of the Triton X-100-solubilized and radioisotope-labeled plasma membrane proteins from the wheat germ lectin-Sepharose 6MB chromatography step were analyzed in a column of immobilized Ad2 virions. Figure 7 shows the elution pattern when unretained material from the lectin chromatography (pool A, Fig. 6) was applied to the column. Of the radioactive material, 95% had no affinity for Ad2 and could be washed out with WGA buffer. Of the remaining 5% of radioactivity, 3% was eluted with WGA buffer containing 0.5 M NaCl, whereas the radioactivity still bound was eluted only after a drastic decrease in pH (see Materials and Methods).

When membrane proteins possessing *N*-acetyl-D-glucosaminyl residues (pool B, Fig. 6) were fractionated on the Ad2 column, a totally different pattern was obtained. Only 41% of the total radioactivity could be washed out unadsorbed with WGA buffer (Fig. 7B). Of the remaining

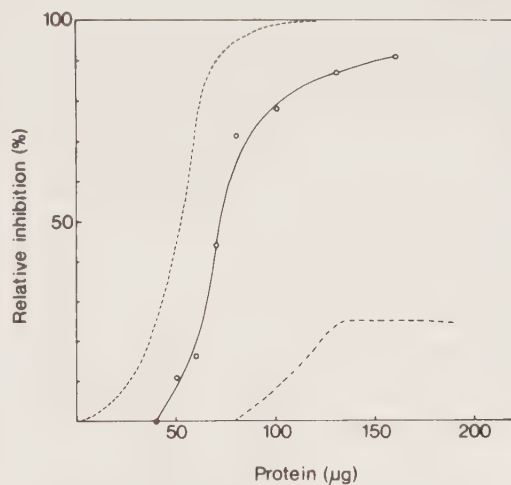


FIG. 3. Relative inhibition of virus attachment to HeLa cells in the presence of various amounts of presumptive receptor-active material. Relative inhibition in the presence of (---) plasma membrane proteins extracted with 0.5% Triton X-100 (the mean function was constructed based on three separate experiments with six measuring points per experiment); (—○—) total plasma membranes of HeLa cells (one experiment was performed); and (-.-.-) dissolved protein content from the nonextractable remainder after a 0.5% Triton X-100 extraction. The mean function was constructed based on two separate experiments with six measuring points per experiment.

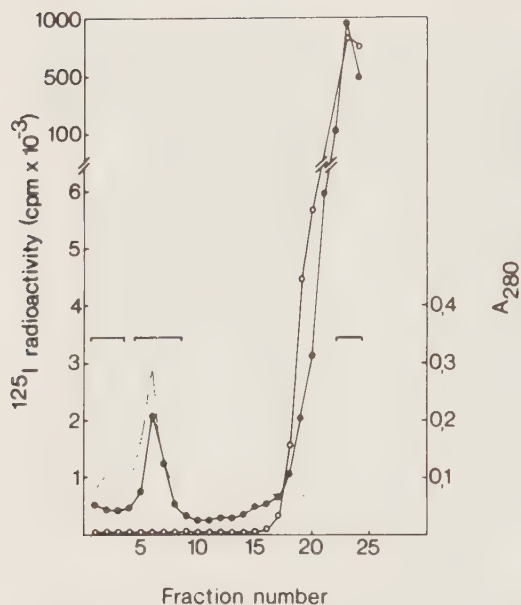


FIG. 4. Sucrose gradient analysis of HeLa cell plasma membrane proteins adsorbed to adenovirus particles. Symbols: (—●—) Radioactivity of Triton X-100 (0.5%)-solubilized plasma membrane proteins, ^{125}I -labeled *in vitro* and mixed with Ad2 virions; (—○—) radioactivity of solubilized, iodine-labeled proteins analyzed for self-aggregation in a separate centrifuge tube; (...) UV absorbance (260 nm) of Ad2 particles sedimented in a separate centrifuge tube as a sedimentation control for the system. A typical experiment with 0.037% Triton X-100 in incubation mixtures and gradients is shown, details of which are described in Materials and Methods. Sedimentation was from right to left. The horizontal bars indicate fractions pooled for further analysis (see Fig. 5).

59%, 38% was eluted at high ionic strength (0.5 M NaCl) and 21% was eluted at low pH (2.6). This means that about one-fifth of the wheat germ lectin-adsorbed proteins had high affinity for Ad2, which further corresponds to about 1% of the totally solubilized membrane protein content. This figure should be compared with the 0.3% of solubilized membrane proteins adsorbed to Ad2 virions in the sucrose gradient system.

Polypeptide analysis of the virus-retaining material. From the virus affinity chromatograms, the different peak fractions were pooled as indicated in the Fig. 7 and further analyzed on SDS-polyacrylamide slab gels (Fig. 8). Because of the low amount of radioactivity applied, patterns for sample wells with several polypeptides were difficult to observe. The pooled fractions from pool B in the lectin chromatography step (Fig. 6) and with high affinity for Ad2 virions (pool B3 of Fig. 7B) revealed one heavily

and one poorly labeled polypeptide band with apparent molecular weights of 42,000 and 40,000, respectively. The analogous fractions derived from pool A (Fig. 6 and 7A) showed two equally labeled polypeptide bands with the same apparent molecular weights as those of pool B3 (Fig. 7B).

DISCUSSION

The plasma membrane fraction of HeLa cells has been isolated preparatively, and by means of various extraction procedures some information has been obtained on the nature of the Ad2 attachment proteins and their association with the plasma membrane.

Assuming that the components that are important for the initial attachment step of viruses to cells are loosely associated surface entities of the plasma membrane, high-salt conditions should be effective in releasing this material. The 3 M KCl extraction procedure used here is well established for these purposes (33), but a high-salt extract of plasma membranes had little effect in the relative inhibition assay (Table 2). Presumably due to denaturing conditions of the high-salt procedure or the removal of a cooperating attachment factor, a consecutive Triton X-100 detergent extract also had little effect in the receptor assay. The results indicate that the attachment proteins are localized as integral entities of the plasma membrane. This is further supported by the fact that removal of the free detergent from a 0.5% Triton X-100 extract decreased the inhibitory activity (Table 2).

That the assayed components were integrated structures of the membranes was further indicated by the observation that an extract of the nonionic detergent Tween 20 did not display any measurable receptor activity (Table 2). One explanation for the lack of receptor release with this detergent could be the higher hydrophilic-lipophilic balance value for Tween 20 (16.7) than for Triton X-100 (13.5). It is generally believed that hydrophilic-lipophilic balance values above 14.5 are associated with lower ability to solubilize integral membrane proteins (15, 17). Of course, the different configurations of the detergents, i.e., size and structure of the polar and apolar moieties of the detergent molecules, could also contribute to the distinct extraction properties. Conformational changes of the attachment proteins cannot be ruled out, but to circumvent this possibility the receptor assay was developed and an extraction procedure was eventually selected that yielded receptor-active material in a soluble state.

Hennache and Boulanger (18) described the isolation of proteins interfering with the attach-

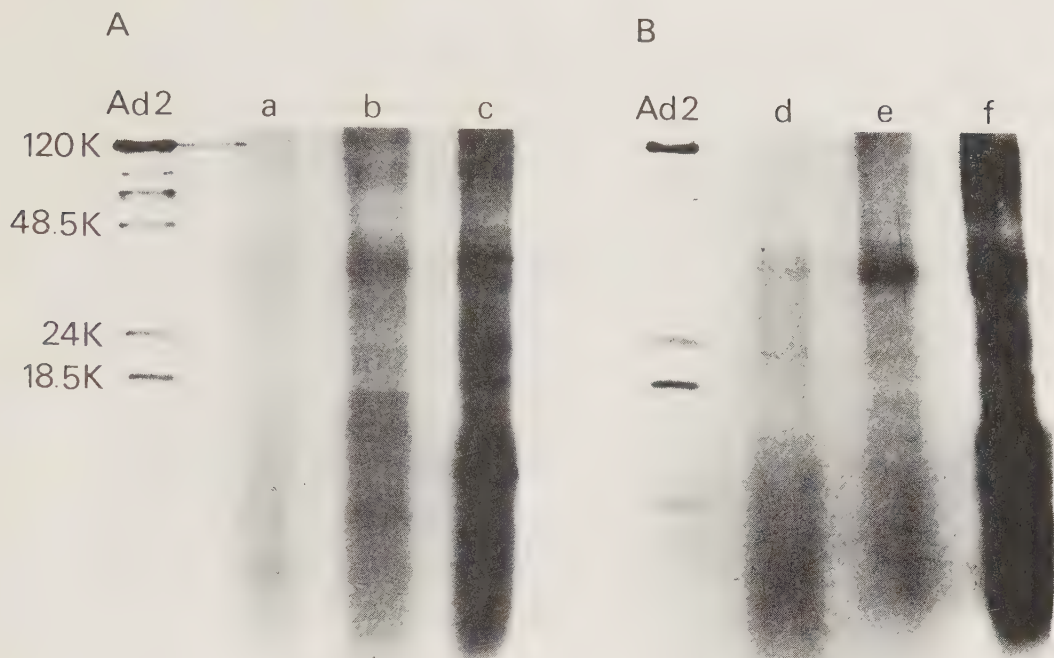


FIG. 5. Autoradiogram of an SDS-polyacrylamide slab gel electropherogram of ^{125}I -radiolabeled membrane proteins recovered from a sucrose gradient sedimentation analysis of proteins adsorbed to Ad2 particles. (A) Analysis of membrane proteins recovered from a sucrose gradient sedimentation in the presence of 0.037% Triton X-100 (Fig. 4). (a) Fractions 1 through 3; (b) fractions 5 through 9; (c) fractions 23 through 24. (B) Sucrose gradient analysis in the presence of 0.5% Triton X-100. Fractions from the sedimentation chromatogram were pooled as in (A), giving sample slots d, e, and f. ^{14}C -amino acid-labeled Ad2 virus was used as a polypeptide marker with indicated positions and molecular weights of major structural proteins as described by Anderson et al. (1). The gels were run as described in the legend of Fig. 1.

ment of Ad2 to KB cells. Fibers and total penton structures of the virions, immobilized by various techniques, were used as adsorbents. By using total Ad2 virions, we have allowed for the subsequent identification of all possible membrane components of stronger as well as weaker affinity for all surface structures of the intact Ad2 virion.

We noticed a quantitative difference in the amount of extracted proteins associated with virions under various conditions. In the sucrose gradient system, the attachment efficiency ranged between 0.2 and 0.3% when Triton X-100 was present at 0.5 and 0.037%, respectively. Membrane proteins were in these instances added in amounts ranging from 2×10^{-10} to 3×10^{-10} μg per virion. Ten- to 100-times-lower ratios of protein over virions were used in the immobilized system, and under these conditions as much as 3% of the solubilized protein content was adsorbed. Of this material, one-third (i.e., 1%) was retained with high affinity. The calculations were based on pertinent fractions, taking

into account all possible technical considerations. Meager et al. (31) and Hughes and Mautner (21) have suggested the involvement of glycoproteins during virus-cell interaction, and our preliminary data also suggest that glycosylated components are of interest. Therefore, fractionation on WGA-Sepharose was done to drastically concentrate the glycoprotein content of the total membrane extracts; in this way, samples relatively rich in receptor activity were obtained for the subsequent fractionations on the immobilized virion particle matrix. It should be emphasized that this prefractionation was not done before the assays in the sucrose gradient system, and this fact could explain the lower yield of virus-attaching proteins in the gradient analyses compared with the Ad2 matrix system. Supporting data for the idea of nonspecific protein competition may also come from experiments where large quantities of added proteins irrelevant for the virus-cell attachment step, e.g., BSA, strongly affected the attachment of membrane

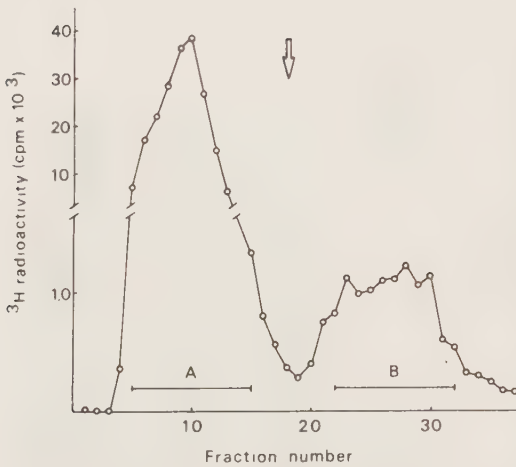


FIG. 6. Wheat germ lectin-Sepharose 6MB chromatography of Triton X-100-solubilized and *in vitro* [^3H]formaldehyde-labeled plasma membrane proteins of HeLa cells. A typical experiment is shown in which 1.4×10^7 cpm of radioactivity was applied to the column and eluted as described in Materials and Methods. The arrow indicates the start of elution with WGA buffer containing *N*-acetyl-D-glucosamine (100 mg/ml). The horizontal bars (A and B) represent fractions pooled for further studies (see Fig. 7).

proteins to virions. These findings are contradictory to the results of Hughes and Mautner (21), who in their not fully comparable system of purified fibers and membrane components were not able to affect the fiber-receptor recognition.

The analyses in sucrose gradients and on immobilized virus particles apparently show at least two types of affinity of membrane proteins to virus particles. We focused here on the high-affinity fraction, but it is possible that proteins with lower affinity for viruses still may be involved in the attachment step. The high-affinity fraction obtained by the two procedures is dominated by a radiolabeled polypeptide with an apparent molecular weight of 42,000. One of the Ad2 attachment polypeptides on KB cells enriched by Hennache and Boulanger (18) displayed the same molecular weight (18).

Based on the elution profiles from the affinity chromatographies on WGA and Ad2 matrices (Fig. 6 and 7), we suggest that the high-affinity proteins contain *N*-acetyl-D-glucosaminyl residues. This means that the indicated molecular weights may be uncertain, because SDS-PAGE is not the optimal system to determine the molecular weight of glycoproteins (14). The autoradiogram patterns for pools A3 and B3 (Fig. 8) were very similar, and the visible bands probably corresponded to the same polypeptide. However, with respect to the different affinities for WGA, the carbohydrate moieties of the polypeptides in

A3 and B3 must have been distinct, since the elution patterns were not caused by overloading artefacts during chromatography. The different behaviors may be due to a partial degradation of the carbohydrate moiety or incomplete glycosylation during the biosynthesis of the polypeptides recovered in pool A3 (28).

Since it has been reported that the receptors for Ad2 are sparsely represented on human cell plasma membranes (21), we have worked with membrane proteins radiolabeled *in vitro* to identify the components of the attachment site. It is not possible to obtain the same specific radiolabeling of all polypeptides in the plasma mem-

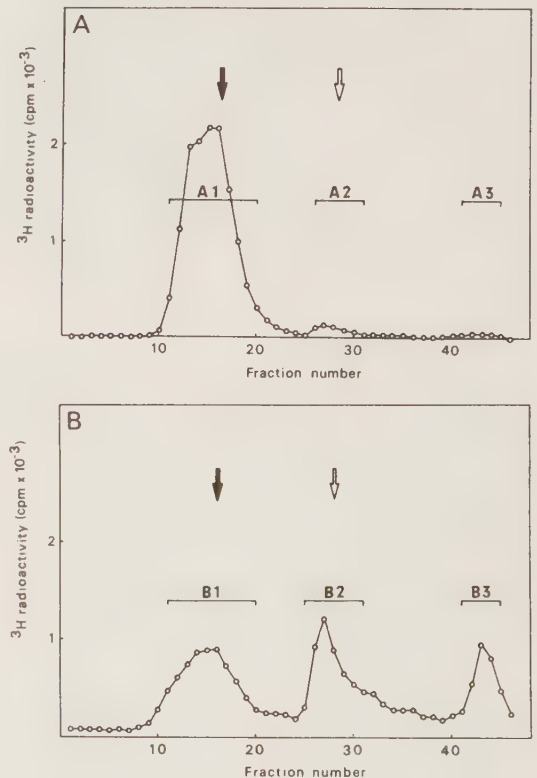


FIG. 7. Affinity chromatography on an immobilized Ad2 matrix. (A) Pool A from the wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was applied to a column of cross-linked Ad2 virions immobilized to AH-Sepharose 4B and fractionated as described in Materials and Methods. The filled arrow indicates the start of elution with WGA buffer containing 0.5 M NaCl, and the open arrow indicates the start of elution with 0.1 M NaCl-0.037% Triton X-100 in 0.1 M glycine-hydrochloride buffer, pH 2.6. The horizontal bars represent the peak fractions pooled and are denoted A1, A2, and A3 according to elution order. (B) Pool B from the previous wheat germ lectin-Sepharose 6MB chromatography separation (Fig. 6) was fractionated as described above.

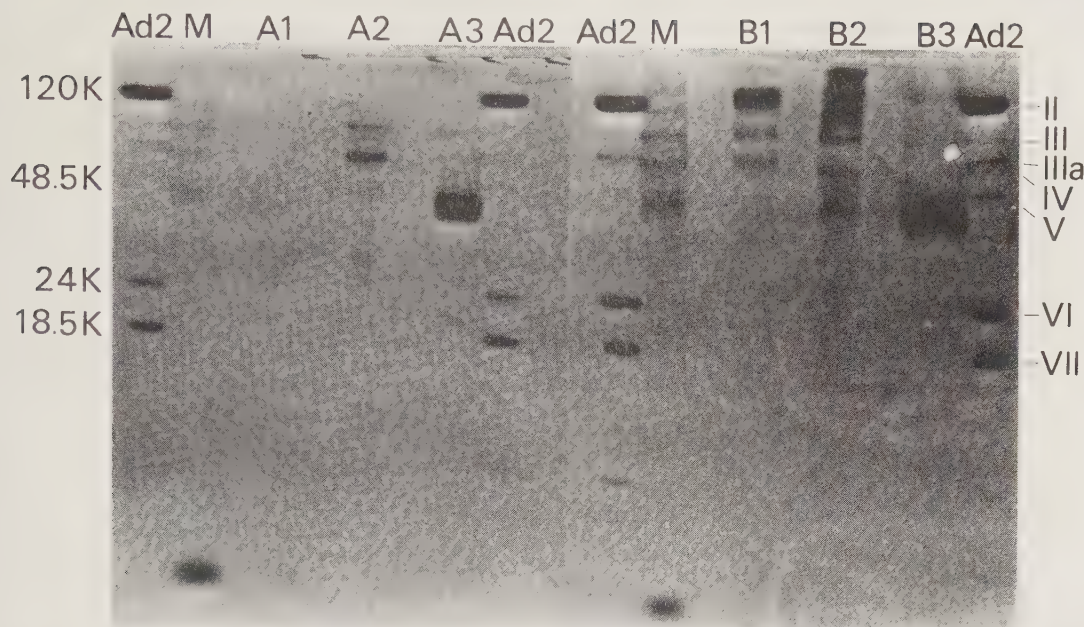


FIG. 8. SDS-PAGE of pooled fractions from an immobilized Ad2 affinity chromatography separation of prefractionated HeLa cell plasma membrane proteins labeled *in vitro*. Samples recovered as described in the legend of Fig. 7 were applied to each sample well in equal amounts of radioactivity (6,000 cpm). Lane M is total, solubilized, and *in vitro* [^3H]formaldehyde labeled plasma membrane proteins. A1, A2, and A3 are the pooled fractions of the separation demonstrated in Fig. 7A; B1, B2, and B3 are the corresponding fractions of the separation of Fig. 7B. A ^{14}C -amino acid-labeled preparation of Ad2 was used as a polypeptide marker with indicated positions and molecular weights of major structural polypeptides as described by Anderson *et al.* (1). The gels were run as described in the legend of Fig. 1 and subsequently analyzed by fluorography.

brane. One must therefore consider that some polypeptides may escape being labeled. Thus, our preliminary results with unlabeled HeLa cell plasma membrane proteins reveal similar patterns when chromatographed on WGA and Ad2 matrices. However, in these instances the A3 and B3 pools, upon SDS-PAGE analysis, display distinct polypeptide patterns after staining of the electropherograms. Moreover, it has been demonstrated that very low quantities of unlabeled proteins of pool B3 interfere with the Ad2 attachment to cells (R. Persson, U. Svensson, and E. Everitt, manuscript in preparation).

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Virus Receptor Interaction in the Adenovirus System II. Capping and Cooperative Binding of Virions on HeLa Cells

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Adenovirus type 2 attachment to HeLa cells was analyzed under controlled conditions. The temperature-dependent attachment kinetics revealed an inflection point at around 20°C, and above this temperature the increase of the rate was doubled. In multiplicity dependence experiments, the attachment exhibited positive cooperative binding at 37°C. This binding pattern was inhibited by low temperatures and prefixation of cells with 0.015% glutaraldehyde. Attachment of rhodamine-labeled virions revealed capping of the particles on 15% of the cells at 37°C. Capping was inhibited by low temperatures, glutaraldehyde fixation of cells, and treatment with cytochalasin B, azide, and 2-deoxyglucose. Consequently, we propose that the adenovirus type 2 attachment to cells leads to rearrangements in the plasma membrane, resulting in cooperative binding and capping of the virus particles.

The important first step for a successful replication of animal virus includes attachment, penetration or internalization, and uncoating. Attachment to cells is believed to involve an interaction between viruses and specific components of the plasma membrane. Lonberg-Holm and Philipson (18) have outstandingly surveyed the early interactions between animal viruses and cells, and review articles which focus on recent achievements within the field continually appear (3, 5, 19).

For clarity, we have adopted the nomenclature defined by Lonberg-Holm (17): virus attachment proteins are the structural components of the virion recognizing a specific cellular receptor, cellular receptor units (CRU) are cellular molecules recognizing one virus attachment protein, and cellular receptor sites (CRS) are cellular structures composed of one or more CRU involved in the binding of a virus particle.

In the adenovirus system, the well-characterized fiber protein has been identified as the virus attachment protein in the sense that isolated fibers adsorbed to cells efficiently block a subsequent virion attachment (26). Moreover, antibodies against purified fiber antigen quantitatively precipitate virions (27) and consequently inhibit infection as assayed by the fluorescent focus technique, but curiously not by the plaque assay (23, 24). The maximum number of virions which can attach to a cell is limited by the number of available CRS. Since in the adenovirus system the CRU/CRS ratio is in the range of 10 to 100 (26), either the isolated fiber molecules

may recognize attachment units unavailable for the binding of larger virions, or virion attachment implies the need for engagement of several CRU. This multivalency of CRS may be achieved during the mere process of virion attachment, which might cause physical alterations of the lipid bilayer, as well as provoke changes in the lipid composition within the microenvironment of the CRS (17).

Kohn (15) has suggested that attachment of viruses or ligands to possibly cytoskeletally anchored CRU cross-links the receptors and thus modifies the interaction of the latter with the lipid phase of the membrane, and this is expressed as a change in fluidity. Interestingly, it has been shown for some nonenveloped viral systems that a decrease in microviscosity is induced upon virus attachment, an attachment-specific phenomenon since it only appeared in cells with intact receptors and in cells permissive with regard to the attachment step (16). As a consequence of virion adsorption to KB cells at low temperatures, Hennache et al. (10, 11) demonstrated that adenoviruses induce a rearrangement and clustering of intramembranous particles and a change in the distribution of sterols.

In light of the preceding information, we have in this study made attempts to unravel the mechanisms behind the adenovirus type 2 (Ad2) attachment to HeLa cells by manipulating the permissive system through changing a physical parameter such as the temperature, chemically stabilizing the cell surface by fixation, and adding physiologically interacting drugs and reagents.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at cell densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenoviruses were propagated, radioisotope labeled, and purified as described previously (7).

Attachment studies. The general procedure of virion attachment was as described earlier (35). HeLa cells were washed in phosphate-buffered saline (PBS) once or three times for experiments in unfixed or glutaraldehyde (GA)-fixed series, respectively. [3 H]thymidine-labeled virions (3.0×10^6 cpm per 10^{12} virions) were allowed to attach to cells in PBS at a density of 5×10^7 cells per ml.

In temperature dependence experiments, cells and viruses were incubated in PBS at temperatures between 2.8 and 37°C. In all experiments, cells were equilibrated at the relevant temperature for 5 min before viruses were added. The number of virions per cell was kept at a constant multiplicity of infection (MOI) of 340. Samples were withdrawn at 5, 10, 20, 30, 45, and 60 min after the addition of virus, diluted 10 times in PBS, and gently sedimented for 25 s. The radioactivity confined to cell pellets and supernatants was measured and used for calculating the degree of virion attachment.

In multiplicity dependence experiments, ratios of viruses over cell numbers were between 50 and 20,000. Incubation periods for cell and virus mixtures were 45 min for unfixed and GA-fixed cells at 37°C and 3 h for unfixed cells at 2.8°C. After incubation, samples were diluted six times in PBS, sedimented, and assayed for radioactivity as described above.

The possible effect of cytochalasin B (15 to 60 μ g/ml) colchicine (0.1 to 0.4 mM), sodium azide (10 to 50 mM), and 2-deoxyglucose (10 to 50 mM) on the virion attachment was studied. Before virus addition, cells were preincubated for 10 min at 37°C with the appropriate reagent. Ad2 was added at an MOI of 340, and incubations were continued for 60 min. Samples were withdrawn at intervals, and virus attachment was assessed as above. The same reagents were used in the fluorescence studies.

GA fixation of cells. Various concentrations of GA were tested to obtain an optimal stabilization of cells with a concomitant minimal influence on the properties of the CRS. Cells, washed three times in PBS, were suspended in PBS at a concentration of 10^6 per ml. Freshly prepared 2.5% GA in PBS was added to yield final concentrations of 0.001 to 0.6%. Cell suspensions were incubated at 4°C for 30 min under slow agitation, after which the cells were sedimented and resuspended, at the density above, in PBS containing 0.05 M neutralized ethanolamine. After a 30-min incubation, cells were washed three times in PBS and finally suspended at a density of 5×10^7 per ml. The status of the cells was studied accordingly.

Virion attachment at 37°C revealed that cells fixed with 0.01% GA were almost unaffected, with a reduction in attachment of less than 10%, whereas at 0.06% GA the reduction amounted to 90%. Swelling of cells in hypotonic buffer (10 mM Tris-hydrochloride buffer containing 10 mM KCl, 2 mM MgCl₂, and 2 mM β -mercaptoethanol, pH 7.3) for 15 min at 22°C, detergent

treatment (0.1% Triton X-100 in PBS) of cells for 60 min at 22°C, and ultrasonic treatment of cells for 5 s on ice demonstrated that cells stabilized with 0.01% GA appeared to be unaffected by the various treatments, as judged from microscopic examination. A concentration of 0.015% GA was chosen in our subsequent fixation studies of cells, and at this concentration virus adsorption was reduced at the most by 30%.

Rhodamine labeling of Ad2 virions. Tetraethylrhodamineisothiocyanate (TRITC) was covalently linked to Ad2 virions as follows. Immediately before conjugation, highly purified virions were filtered through a PD-10 column, equilibrated in 0.1 M NaHCO₃, pH 8.7. The concentration of recovered viruses was 3.6 optical density units per ml, corresponding to 1.01 mg of virus protein (22). TRITC in 0.1 M NaHCO₃-ethanol (1:1) at a concentration of 1.0 mg/ml was added to the virus suspension at a ratio of TRITC to virus protein of 0.05. The mixture was incubated at 26.5°C for 3 h, after which the conjugated virions were separated from free TRITC in a PD-10 column equilibrated in PBS, with a recovery of 75%. Spectrophotometric measurements at 560 nm revealed a number of TRITC molecules per virion in the range of 5×10^3 to 7×10^3 . No disintegration of conjugated virions was discernible after sedimentation analyses in glycerol gradients. The capacity of TRITC-labeled Ad2 to attach to cells was reduced by 10 to 15%, and unconjugated Ad2 particles competed quantitatively with TRITC-labeled Ad2 for receptor sites on the cells.

Attachment studies with TRITC-conjugated Ad2. Cells were carefully washed and suspended in PBS at a density of 5×10^7 per ml. TRITC-conjugated Ad2 was added at an MOI of 3,000 and adsorbed at 2.8°C for 60 min. After attachment, cells were washed with PBS and further processed in one of the following ways.

(i) Cells were fixed with GA directly after the initial attachment step. (ii) cells were incubated further at 37°C for 0.5, 1, 2, 5, 10, 20, 40, and 60 min and subsequently fixed. (iii) Cells were fixed and further incubated at 37°C for 60 min. TRITC-conjugated Ad2 was also added to prefixed cells and incubated at 2.8°C for 60 min.

In addition, attachment of TRITC-conjugated Ad2 was studied in the presence of sodium azide (10 and 30 mM), cytochalasin B (15 μ g/ml), colchicine (0.1 mM), and 2-deoxyglucose (10 mM). The virions were added to cells together with one of the reagents and incubated for 60 min at 2.8°C. After removal of unadsorbed virus with PBS, cells were suspended in PBS containing the indicated concentrations of reagents and further incubated at 37°C for 10 min. Immediately after the second incubation, the cells were fixed with 0.015% GA. Analyses were made in a Zeiss fluorescence microscope by using epifluorescence with BP 546, FT 580, and LP 590 filters. For photography, Ilford HP 5 films were used.

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were done in a cocktail of toluene-methanol (1:1) with 0.4% Omnifluor. A Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) was used to determine radioactivity.

Chemicals and reagents. Eagle minimal essential medium, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. [3 H]thymidine (74 Ci/mmol) and Omnifluor

were purchased from New England Nuclear Chemicals GmbH, Dreieich, West Germany. Cytochalasin B, colchicine, sodium azide, and 2-deoxyglucose were from Sigma Chemical Co., St. Louis, Mo. Rhodamine B isothiocyanate and ethanolamine were obtained from BDH Chemicals Ltd., Poole, England, and GA (25% for electron microscopy) was from E. Merck AG, Darmstadt, West Germany. PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

RESULTS

Temperature dependence of Ad2 attachment.

The attachment of Ad2 to cells was analyzed at several temperatures ranging between 2.8 and 37°C. To minimize fluctuation effects due to the cellular status, all samples were analyzed within 1 day and with material obtained from the same cell harvest. The virus attachment was highly dependent on the incubation temperature (Fig. 1). Also depending on the incubation temperature, maximum values of attachment were reached after different periods of incubation. These maximum values varied from around 80% attachment after 30 to 40 min at 37°C to around 45% after 3 h at 2.8°C (data not shown).

From the slopes of the curves in Fig. 1, the initial rates of viral attachment were calculated. Figure 2 shows that the attachment rates were strongly dependent on the incubation temperatures, with a shift at around 20°C, leading to a doubling of the rate increase above this temperature.

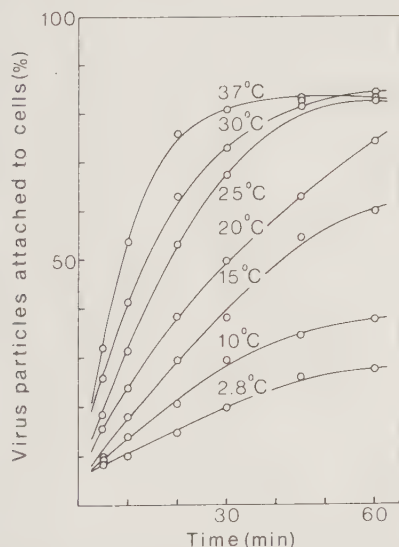


FIG. 1. Temperature dependence of the Ad2 attachment kinetics. Radioactive Ad2 virions were added to cells equilibrated at the appropriate temperature and further incubated. Samples from each incubation mixture were withdrawn at the indicated times, and the attachment of virions was determined as described in the text. The circles represent the mean values of attached viruses in two separate experiments.

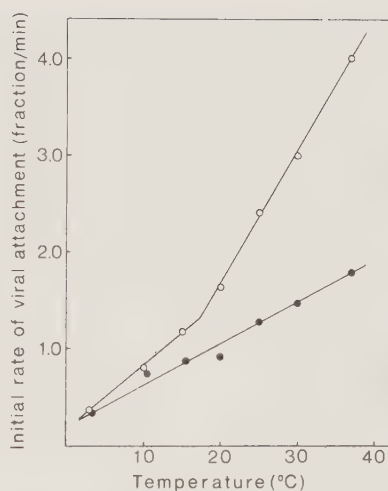


FIG. 2. Temperature dependence of the initial rate of adenovirus attachment. The initial rates of viral attachment at the different temperatures were calculated from the slopes in Fig. 1 to give the fraction of adsorbed virions per minute (○). ●, Attachment data from an analogous experiment with GA-fixed (0.015%) cells.

ture. For comparison, an analogous curve was made for cells fixed with 0.015% GA. The slope of this curve resembled the one for unfixed cells below 20°C, but revealed no shift in the increase in attachment rates above 20°C.

Temperature dependence of attachment of rhodamine-labeled Ad2. Ad2 particles conjugated with the isothiocyanate derivative of rhodamine were employed to visualize the early events of virion attachment. Due to the high specific fluorochrome labeling of virions, MOIs of 3,000 particles per cell were used to give a moderate number of attached virions (ca. 20%). Cells incubated with TRITC-conjugated Ad2 at 2.8°C for 60 min and subsequently fixed with 0.015% GA demonstrated a granular and patchy fluorescence pattern, uniformly distributed over the cell surface (Fig. 3A). This general pattern was not altered if fixed cells were transferred to 37°C and further incubated for 60 min.

The same pattern was also observed if cells were fixed before virion attachment and incubated as above. However, if cells with virions adsorbed at 2.8°C were transferred to 37°C, without prior fixation, the fluorescence became clustered and polarized to one end of the cells at a frequency of around 15% (Fig. 3B and C). Capping of labeled virus was evident as early as 30 s after the temperature shift. Approximately 20 min after the shift, the fluorescent clusters appeared less pronounced and became somewhat diffuse (Fig. 3D), possibly due to translocation of virions into the cytoplasmic region of the cells.

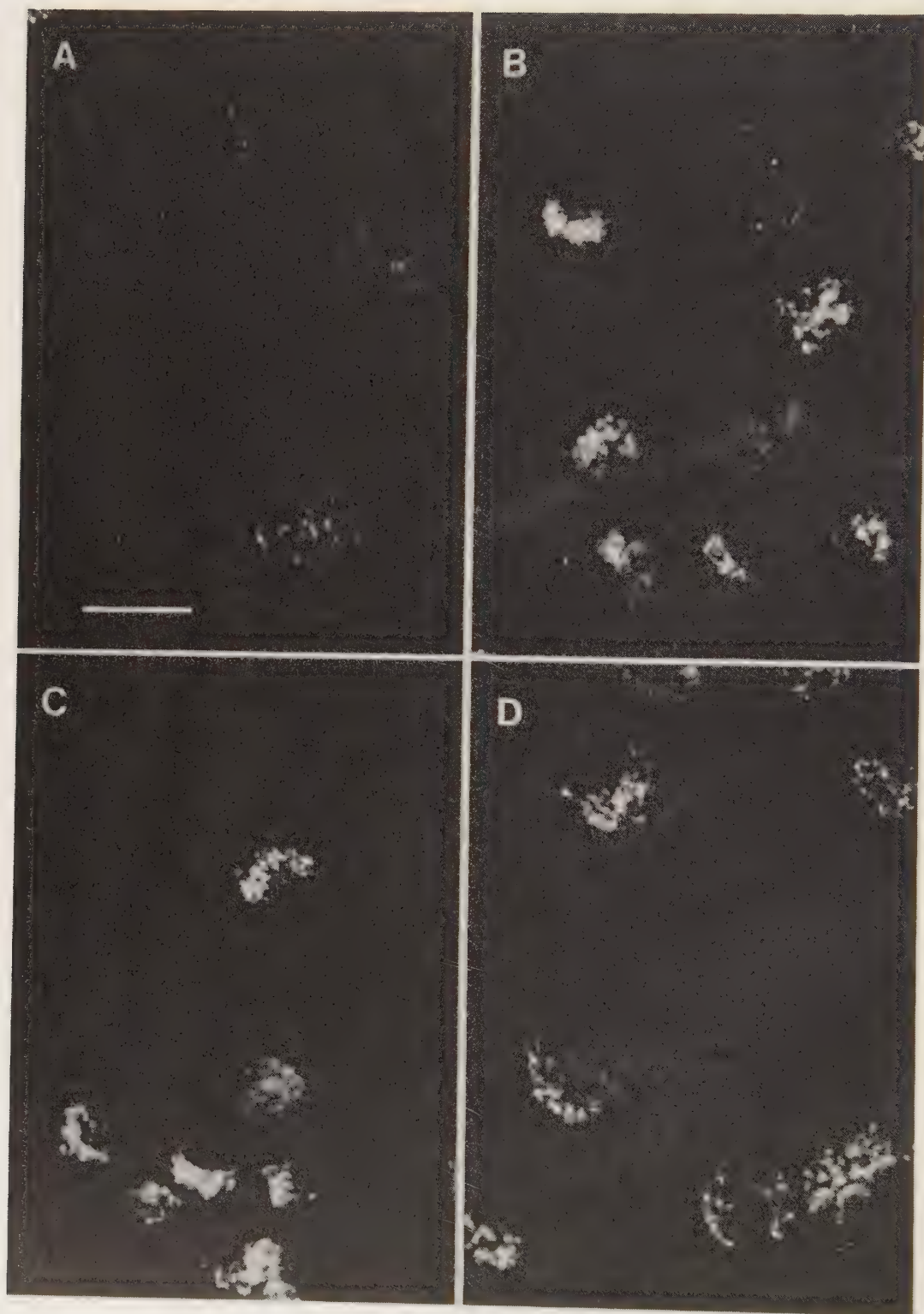


FIG. 3. Attachment of rhodamine-labeled Ad2 virions. Rhodamine-labeled virus was added to cells at an MOI of 3,000 at 2.8°C for 60 min. After removal of unadsorbed virus (80%), cells were transferred to 37°C and incubated for increasing periods of time. Samples were removed, subsequently fixed with GA (0.015%), and

Influence of drugs and metabolic inhibitors on virion attachment. Metabolic inhibitors (NaN_3 and 2-deoxyglucose) and drugs interfering with the cytoskeletal elements (cytochalasin B and colchicine) were analyzed with respect to possible effects on virion attachment. The reagents were added to cells 10 min before the addition of radiolabeled virus, and attachment was continually measured during a period of 60 min at 37°C. Azide, cytochalasin B, and colchicine were negative or reduced the final attachment by less than 10%, whereas 2-deoxyglucose at 50 mM reduced attachment by 20% as compared with control cells.

Although exhibiting only minor effects on viral attachment kinetics, the above reagents were also used to study the possible influence on the distribution of virions attached to the cell surface. In these instances, the reagents were added to cells at 2.8°C and incubated along with the TRITC-labeled virions for 60 min. After removal of unadsorbed virus with PBS, reagents were added again before the cells were transferred to 37°C for 10 min. After the second incubation, all cells were fixed with 0.015% GA, and 300 cells from each series were examined. The already-mentioned extent of capping to around 15% in unfixed cells at 37°C was inhibited after the addition of sodium azide (10 and 30 mM), cytochalasin B (15 $\mu\text{g/ml}$), and 2-deoxyglucose (10 mM) and thus equalled the background level of 2% for cold-treated or GA-fixed cells. Colchicine treatment of cells (0.1 mM) had no effect on capping.

MOI dependence of Ad2 attachment. Ad2 was added to HeLa cells at different MOIs ranging from 50 to 20,000 virions per cell. The virus-cell mixtures were incubated at 37 and 2.8°C for 45 min and 3 h, respectively. Virus attachment was studied on both GA-fixed and unfixed cells. Figure 4 shows the number of bound viruses at various MOI inputs. The curves reveal typical saturation patterns, demonstrating the virus-binding capacity of each cell. In this particular experiment, the GA fixation reduced the number of available CRS to 60% of the control cells. This was an exceptional outcome of the GA treatment, which normally did not display this CRS-reducing effect. However, these results are shown for the sake of comparison, since all temperature- and MOI-dependent experiments described in this communication were derived from the same cell harvest and performed within 1 day.

After conversion of the results into Scatchard plots, a positive cooperative binding at virion

attachment was reproducibly discernible for untreated cells at 37°C (Fig. 5A). No such effect was revealed for the GA-fixed cells or for the attachment system at 2.8°C (Fig. 5B). A positive cooperative binding was detected in all experiments performed with untreated cells at 37°C. However, the position and width of the region of the function indicating this effect varied. An obvious explanation for this variation is the fluctuation in the status of the cells. From the Scatchard plots the numbers of CRS were estimated, and values ranging between 3,000 and 6,000 CRS per cell were obtained for all three series studied.

The Scatchard plots were further used for calculations of the association constants, and on a per-receptor-site (CRS) basis, a value between 0.8×10^{10} and $1.2 \times 10^{10} \text{ M}^{-1}$ was obtained for the virus-cell interaction at 37°C, whereas for the other two series the constants were in the range of 0.3×10^{10} to $0.4 \times 10^{10} \text{ M}^{-1}$.

DISCUSSION

In this study we have specifically focused on the early interactions occurring between human Ad2 and CRS of the HeLa cell plasma membrane during the phase of virion attachment.

Experiments designed to study the temperature dependence demonstrated that the initial rates of viral attachment were strongly temperature dependent, as previously indicated by Philipson (25) and Inoye and Norrby (12). A shift in the rate increase by a factor of 2 was established for temperatures above 20°C. The reproducibly observed inflection point of the kinetics curve might be due to increased possibilities for the CRS to move laterally in the plasma membrane. This could be a consequence of alterations in the physical status of the lipid matrix, such as gel-to-liquid crystalline phase transitions or phase separations (2) or both. Besides an increased movement, alterations in the lipid moiety would allow a hypothetical unmasking of individual units within the receptor sites, further leading to higher affinity for virus particles. These ideas emerging from temperature dependence experiments are likewise supported by the results obtained with GA-fixed cells. The chemical fixation or cross-linking will lead to an increase in the rigidity of the membranes, where proteins no longer can undergo conformational changes or diffuse freely in the membrane (4, 21, 30). When our data were converted into Arrhenius plots (not shown), the activation energy of virus attachment was shown to be around 42 kJ/mol at temperatures above the inflection point, where-

studied in a fluorescence microscope. (A) cells fixed with GA directly after incubation at 2.8°C; (B to D) cells fixed with GA after transfer to 37°C and a further incubation for 0.5 (B), 5 (C), and 20 (D) min, respectively. All photographs are of the same magnification; bar = 25 μm .

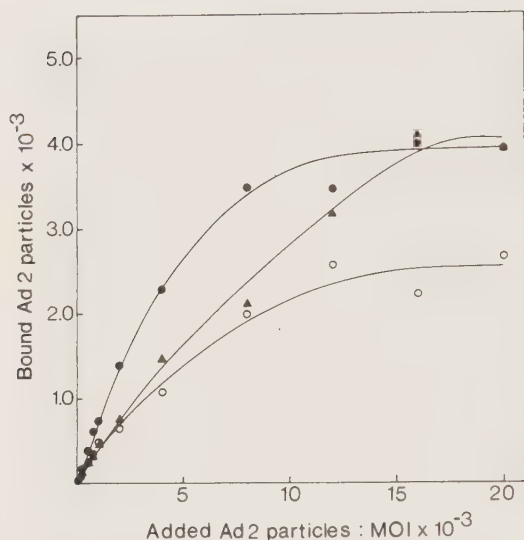


FIG. 4. Multiplicity dependence of the adenovirus attachment. Ad2 particles were added to cells at different MOIs, and the number of attached virions was estimated after different periods of incubation: ●, at 37°C for 45 min; ▲, at 2.8°C for 3 h; and ○, GA-fixed cells at 37°C for 45 min. For details, see the text.

as about 59 kJ/mol was obtained for the curve below this point, again indicating a temperature-dependent alteration of the system. In analogous attachment experiments with human rhinovirus type 2 and poliovirus type 2, linear Arrhenius plots were obtained, with activation energies of around 54 kJ/mol (20). Provided that the cells were pretreated at 0°C for more than 3.5 h, the viral attachment kinetics revealed an inflection point at 18°C. An explanation suggested for this effect was the introduction of the receptor sites into a fluid surrounding, as a consequence of phase separations. In analogy to the above shifts in activation energies at distinct temperatures, comparable effects at around 25°C have been demonstrated for, e.g., lipid analog diffusion in plasma membranes of human fibroblasts (13).

In the experiments with different MOIs, we applied the analysis technique of Scatchard (29) to estimate the number of virus receptor sites per cell and the association constants between viruses and cells at steady-state conditions. The number of CRS for adenoviruses on the HeLa cells was in the range of 3,000 to 6,000, which is in good agreement with previous determinations (26). The number of CRS was not affected by different temperatures of the virus-cell mixtures, and fixation of cells with GA also displayed no or minor effects on CRS numbers. A positive cooperative binding is a well-established phenomenon when multivalent ligands are attached to cells (14, 28), and Scatchard plots for untreated

cells and viruses at 37°C indicate such a cooperative binding at Ad2 attachment. Low temperatures and chemical stabilization by fixation of cells clearly inhibit this effect. In support of our latter finding, it has been reported for other systems that cells fixed with minute amounts of GA do not bind ligands in a cooperative way, as is the case otherwise (32). In addition to the viral multivalency, it is possible that the mechanisms to achieve the demonstrated binding pattern involve either an increase in the affinity of the CRS for viruses or an increase in the number of available binding sites within the CRS. Even though it is pointed out above that the number of CRS is unchanged whether a positive cooperative binding is obtained or not, there is a possibility for unmasking each unit within the CRS, leading to higher affinity of viruses for these sites. The association constants show that cooperative binding is a result of an increased affinity for viruses. The constant for untreated cells at 37°C is two to three times higher than that obtained for cells incubated at 3°C or for GA-fixed cells.

Schlessinger et al. (31) have suggested that the lateral aggregation of membrane components forming the caps and the subsequent immobilization are caused by a ligand-induced conformational change of the receptor molecules or by receptor-ligand complexes that in turn increase the binding affinity between mobile receptors. Our experiments with rhodamine-labeled Ad2 show that adsorbed viruses redistribute in the membrane. Thus, after 10 min at 37°C, about 15% of the cells displayed capping of viruses. Azide, cytochalasin B, and 2-deoxyglucose treatment of cells inhibited the capping, as did low incubation temperatures and prefixation of cells with GA. The methods employed to obtain inhibition of the capping effect have been described for several other model systems (33), and we therefore conclude that the phenomenon studied in our system is a true capping. The observation that only 15% of the cells exhibited caps in our experiments might be explained by the fact that cells with high rates of endocytotic activity have a lower tendency to form caps than do cells with lower degrees of endocytosis (34). Thus, capping might be regarded as a consequence of competition between a lateral aggregation in the membrane and internalization of the ligand. Capping of other nonenveloped animal viruses has been reported for the systems of mengovirus (8), frog virus type 3 (1), reovirus type 3 (6), and also for the mycoplasma-specific bacteriophage L3 (9). Mengoviruses capped on Erlich ascites tumor cells are rapidly internalized (8), and reports concerning other model systems clearly demonstrate that capped ligands are subsequently internalized (33). However,

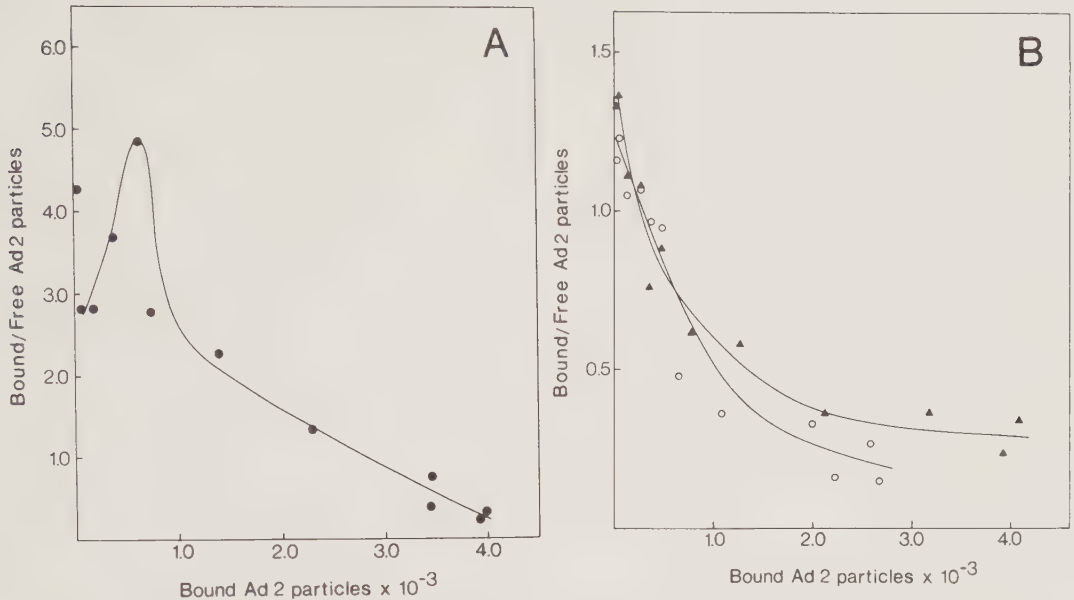


FIG. 5. Scatchard analysis of adenovirus attachment to HeLa cells. The attachment data of Fig. 4 were converted into Scatchard plots. Symbols: ●, virus-cell interaction at 37°C for 45 min; ▲, virus-cell interaction at 2.8°C for 3 h; and ○, virion attachment to GA-fixed cells at 37°C for 45 min. Note the different scales of the ordinates.

the relevance of capping for the following steps of internalization and uncoating in the adenovirus system is at this point speculative, but we are at present studying some aspects of these early events.

In this work, we present suggestive evidence that Ad2 attachment to HeLa cells leads to such alterations in the plasma membrane that redistribution of the viral receptors and possibly configurational changes of the entire receptor sites occur. These concomitant events result in cooperative binding and capping of attached virions.

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Entry of Adenovirus 2 into HeLa Cells

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Adenovirus 2 (Ad2) uncoating was analyzed as the destabilization of virions which renders the parental genome sensitive to DNase treatment. This event demonstrated a strong temperature dependence, and an Arrhenius plot of initial uncoating rates revealed an inflection point at around 16°C. Activation energies of 331 kJ/mol below and 88 kJ/mol above this temperature were obtained for the uncoating process. Penetration of Ad2 through the plasma membrane was completely inhibited by sodium azide, whereas uncoating was only slightly influenced. This indicated that uncoating had already taken place at the outside of the plasma membrane. Incubations of Ad2 with isolated plasma membranes and cell homogenates showed that intact and metabolizing cells were required for uncoating. We further suggest, based on the inhibitory patterns of EDTA, EGTA, dansylcadaverine, and dithiothreitol, that this destabilization of virions follows upon reorganization in the plasma membrane. In the electron microscope the involvement of coated vesicles was shown for the initial uptake of virions, possibly followed by the engagement of acidic vesicles as judged from the effects of lysosomotropic agents on gene expression. The vectorial transport of virions from the plasma membrane to the nucleus was not affected by reagents interfering with the cytoskeletal system. Consequently, we propose that Ad2 virions are internalized by adsorptive endocytosis.

During the past 20 years several investigations have been reported concerning the early interactions between animal viruses and host cells. Each is an important piece in unraveling the question of how animal viruses gain access to host cells. Continuously, the different reports have been brought together in review articles (5, 10, 22, 25).

Carefully conducted investigations have been performed on different enveloped viruses, e.g., Semliki Forest virus, fowl plaque virus, and vesicular stomatitis virus (27-29). These studies show that the virus particles are internalized by adsorptive endocytosis. The envelopes of internalized particles thereafter fuse with membranes of endocytotic vesicles, a mechanism induced by low pH (41). For some members of the enveloped virus families *Herpesviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Poxviridae*, fusion of virion membranes and plasma membranes has been demonstrated as the major mechanism for viral entry (6). Both endocytotic uptake and direct penetration through the plasma membrane have been suggested as internalization routes for nonenveloped viruses (25). It has been stated that reoviruses, once inside the cell, utilize lysosomes to mediate uncoating in the productive cycle (37). For adenoviruses 2 and 5, destabilization in vitro is not achieved in the presence of isolated lysosomal enzymes, implying a nonlysosomal involvement for this process (1).

Adenovirus attachment to specific cellular recognition units leads to redistribution of the cellular recognition units on the cell surface (32, 33). This process has been suggested to facilitate viral internalization (32). The viral capsid is destabilized in close connection with the latter event, leading to a DNase-sensitive viral genome (24, 34, 38). Whether adenoviruses pass through acidic vesicles, before genome expression in the nucleus, is still unknown. However, the engagement of cytoplasmic vesicles has been observed, since adenovirus 2 (Ad2) mediated enhancement of the toxicity of *Pseudomonas* toxin and toxin conjugated to the epidermal-growth factor, by inducing a release of the toxin from intracellular vesicles (12).

In the present investigation we have studied the early

interaction between Ad2 and HeLa cells, by physical and chemical manipulations of the steps connected with internalization of virions. To be certain that we studied the infectious fraction of virus particles, the effect on gene expression was also followed.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenovirus was propagated, radioisotope (^3H)thymidine labeled, and purified as described previously (11).

Attachment studies. The general procedure of virion attachment was as described earlier (39). HeLa cells, washed once in phosphate-buffered saline (PBS) and suspended in PBS at a density of 5×10^7 cells per ml, were infected with [^3H]thymidine-labeled virions (3×10^6 cpm per 10^{12} virions) at a multiplicity of infection (MOI) of 340. The possible effect on the attachment step was studied in the presence of the following reagents: sodium azide (10 to 50 mM), 2-deoxyglucose (10 to 50 mM), sodium azide (10 to 50 mM) together with 2-deoxyglucose (10 to 50 mM), dithiothreitol (DTT) (2 to 10 mM), EDTA (5 to 20 mM), ethylene glycol-bis(β -aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) (5 to 20 mM), cytochalasin B (15 to 60 μ g/ml), colchicine (0.1 to 0.4 mM), trifluoperazine (10 to 50 μ M), dansylcadaverine (0.1 to 1.0 mM), ammonium chloride (10 to 40 mM), chloroquine (0.1 to 0.4 mM), and methylamine (10 to 40 mM). Cells were equilibrated 10 or 30 min before virus addition at 37°C with the various reagents, which were maintained during virus attachment. This step was followed for 60 min.

Isolation and radioisotope labeling of antibodies. The immunoglobulin fraction from a serum raised in rabbits against freeze-thawed (FT) Ad2 particles was ammonium sulfate precipitated and affinity purified on an Ad2-AH-Sepharose 4B column. The Ad2-AH-Sepharose was prepared as previously described (39). The purified virion-specific immunoglobulin, anti-FT-Ad2 immunoglobulin G, was ^{125}I -labeled with 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen), as described by Fraker and Speck (13).

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The specific radioactivity of the labeled immunoglobulin was 1.0×10^8 to 1.9×10^8 cpm/mg of protein.

Virus penetration. Cells were washed once, resuspended in PBS at a concentration of 5×10^7 cells per ml, and subsequently treated with reagents as described above. Virus was added at an MOI of 10,000, and the cell-virus mixtures were incubated at 3°C for 45 min, resulting in ca. 2,500 attached particles per cell. To remove unattached virions, the cells were washed twice and resuspended in PBS at a concentration of 5×10^7 cells per ml, after which the suspensions were incubated at 37°C for 40 min. These incubations were ended by cooling the suspensions to 3°C. Anti-FT-Ad2 [125 I]immunoglobulin G was added at a calculated ratio of 2,000 molecules per attached virus particle, and the incubations were continued for 1 h at 3°C. Free immunoglobulin G was removed by two cycles of washing in PBS, and the amounts of immunoglobulin still associated with the cells were determined. The maximum and minimum values of the virus that remained detectable on the outside of the cells were determined from two series of cells with attached virus; one was kept at 3°C throughout the entire incubation procedure, and the other was kept at 37°C. Nonspecific adsorption of anti-FT-Ad2 [125 I]immunoglobulin G was determined for mock-infected cells in the presence of the various reagents.

DNase sensitivity of the viral genome. The procedure for DNase sensitivity determination was essentially as described by Joklik (21). Cells were processed as above, and [3 H]thymidine-labeled virus was added at an MOI of 1,000. After attachment for 5 min at 3°C, cells were diluted five times, after which unattached virus was removed by sedimentation of the cells. In temperature experiments infected cells, at a density of 10^7 cells per ml in Eagle minimal essential medium, were further incubated at 3, 10, 15, 20, 25, 27.5, 30, 34, and 37°C. Samples were withdrawn after 1, 5, 10, 20, 40, and 60 min, and cells were sedimented and resuspended at a concentration of 5×10^6 cells per ml in 10 mM sodium phosphate buffer, pH 7.5, containing 10 mM $MgCl_2$. The samples were kept on ice until sonicated four times at 15 s each. A volume of 450 μ l of each homogenate was treated with 1 mg of DNase I for 30 min at 37°C, and undegraded [3 H]DNA of virions was precipitated with trichloroacetic acid at a final concentration of 10%. The background level of spontaneous viral nucleic acid degradation was measured by direct precipitation of parental DNA without any DNase treatment. Trichloroacetic acid precipitates were pelleted, washed with ethanol, and dissolved in Protosol. Collected supernatants were neutralized with sodium hydroxide, and radioactivity was measured along with dissolved pellets.

To study the effect on viral capsid destabilization of the reagents listed above, these reagents were added and incubated together with cells in PBS at a density of 5×10^7 cells per ml for 30 min at 37°C before virus infection. Virus was added at an MOI of 340 and allowed to attach for 5 min at 37°C. Unattached virions were removed, and the cells were resuspended in PBS supplemented with the appropriate reagents. Samples were withdrawn from incubation mixtures maintained at 37°C for a total of 40 min.

Reagents demonstrating an inhibitory effect on the kinetics of viral nucleic acid DNase sensitivity were analyzed with respect to the reversible nature of the inhibition. Thus, agents were removed after 40 min of incubation at 37°C, and cells were subsequently incubated for 2 h at 37°C. No differences in the levels of maximum DNase sensitivity were obtained when infections were made with multiplicities of

340 and 1,000 particles per cell at 37°C and 3°C, respectively.

In vitro destabilization of adenovirions was analyzed in the presence of isolated plasma membranes (4, 20) or total cell homogenates, the former together with 10 mM concentrations of $MgCl_2$, $CaCl_2$, or both and of ATP, GTP, or both. Virions were added to the plasma membranes and cell homogenates in calculated quantities yielding MOIs of 24 or 340 virions per cell equivalent. In some experiments incubation mixtures were supplemented with cytoplasmic extracts.

Virus polypeptide production. The production of an early and a late viral antigen, the 72,000-molecular-weight DNA-binding protein (DBP) and the fiber protein, respectively, was analyzed in the presence of the reagents listed above. Cells were pretreated with the various reagents, and virus was added at an MOI of 1,000 and attached for 40 min at 37°C. Reagents and unattached viruses were removed, and cells were diluted to 4.5×10^5 cells per ml in Eagle minimal essential medium containing 5% fetal calf serum and incubated for 30 h. Samples were withdrawn at different times, and cells were sedimented and disrupted in 50 mM Tris-hydrochloride buffer, pH 8.1, containing 1% Triton X-100, for 40 min at room temperature. This treatment was sufficient to give maximum yields of both DBP and fiber protein (data not shown). Cell debris was sedimented at $3,800 \times g$ for 10 min, and supernatants were analyzed for the two viral antigens by rocket immunoelectrophoresis (23) in agarose gels containing monospecific antiserum against each protein.

During the 30-h incubation period, cell samples were assayed for total numbers and viability by the trypan blue exclusion method. No differences were discernible between controls and cells treated with reagents.

Electron microscopy. Cells were harvested, washed with PBS, and incubated in the presence of different reagents as described above. After infection with Ad2 at an MOI of 5,000 and further incubation for 20 min at 37°C, cells were washed, suspended in PBS, and kept at 0°C at a density of 10^6 cells per ml. Fixation was made in 0.015% glutaraldehyde for 30 min at 4°C in a roller bottle as described by Persson et al. (33). After two cycles of washing in PBS, cells were postfixated and dehydrated essentially as described by Ryter and Kellenberger (36) and embedded in Epon.

Protein determination. Protein was determined by the method of Hartree (15) with bovine serum albumin as the standard.

Liquid scintillation spectrometry. Radioactivity measurements in the DNase assay of supernatants and pellets dissolved in Protosol were performed in a cocktail of toluene-methanol (1:1) containing 0.4% Omnifluor. All other radioactive samples were analyzed in Ready-Solv HP/b.

Chemicals. Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories Ltd., Irvine, Scotland. Omnifluor, Protosol, and [3 H]thymidine (75 Ci/mmol) were purchased from New England Nuclear Chemicals GmbH, Dreieich, Federal Republic of Germany. Agarose type I, bovine serum albumin, chloroquine, colchicine, cytochalasin B, 2-deoxyglucose, DNase I (from bovine pancreas), DTT, EGTA, monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemical Co., St. Louis, Mo. Ammonium chloride, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic of Germany. PD-10 columns with Sephadex G-25M and AH-Sepharose 4B were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodogen) was from Pierce Chemical Co., Rockford, Ill. Triton X-100 (scintillation grade) was from BDH, Poole, England. Ready-

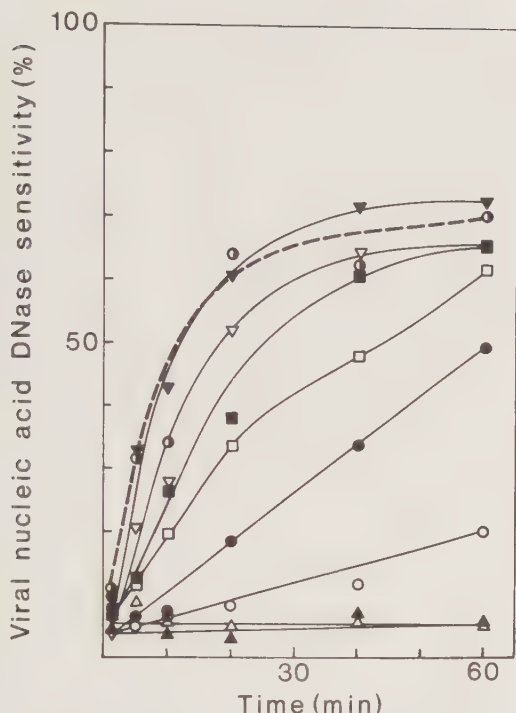


FIG. 1. Temperature dependence of the Ad2-uncoating kinetics. Cells with attached [^3H]thymidine-labeled virus particles (at 4°C for 5 min) were incubated at the indicated temperatures. Samples from each incubation mixture were withdrawn at intervals, and viral uncoating was determined as described in the text. Symbols: Δ , 3°C ; $\blacktriangle$, 10°C ; $\circ$, 15°C ; $\bullet$, 20°C ; $\square$, 25°C ; $\blacksquare$, 27.5°C ; ∇ , 30°C ; $\blacktriangledown$, 34°C ; and $\circ$, 37°C .

Solv HP/b was obtained from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Temperature dependence of Ad2 uncoating. Early in infection, virions of the inoculum were destabilized rendering the parental viral genome sensitive to treatment with exogenously added DNase. We have adopted the word uncoating for this process. The uncoating of cell-attached virions was analyzed at temperatures between 3 and 37°C . Uncoating was strongly temperature dependent (Fig. 1). The initial rates of viral uncoating, at the different temperatures, were calculated from the slopes in Fig. 1. At temperatures below 15°C , uncoating was barely detectable, whereas above 20°C , the uncoating rates increased rapidly to reach a maximum at around 34°C (Fig. 2). The data of Fig. 1 were converted into an Arrhenius plot (inserted in Fig. 2), and a break in the curve at ca. 16°C was obtained. The activation energies for the uncoating process were calculated to 88 kJ/mol above 16°C and 331 kJ/mol below this temperature.

Viral attachment in the presence of various reagents. Before virus internalization and uncoating were studied in the presence of different reagents, these reagents were added to virus-cell mixtures to monitor any possible effects on the attachment step. A concentration of 0.1 mM dansylcadaver-

ine displayed no effect, whereas concentrations of 0.5 and 1.0 mM resulted in inhibitions of attachment of 12 and 41%, respectively. 2-Deoxyglucose (10 to 50 mM) reduced attachment by ca. 20%, and the combination of sodium azide and 2-deoxyglucose resulted in the same inhibition as did 2-deoxyglucose alone. The influence on attachment of all the other reagents was considered negligible ($<10\%$). The data above is in full accordance with the effects of sodium azide, 2-deoxyglucose, cytochalasin B, and colchicine that have been described previously (33).

Influence of various reagents on viral penetration. Affinity-purified ^{125}I -labeled immunoglobulins, against FT-Ad2 particles, were used to determine the amount of attached Ad2 unable to penetrate the plasma membrane of HeLa cells treated with different reagents. Sodium azide (50 mM) inhibited the penetration of Ad2, indicating a dependence of metabolic energy (Fig. 3A). A reduced concentration of azide (10 mM) still displayed the same severe impairment of penetration. Another metabolic inhibitor, 2-deoxyglucose, did not show any inhibitory effect. EDTA and EGTA both reduced the amount of internalized Ad2 to about 25% as compared with internalization in control cells, pointing out the importance of bivalent cations for this process. The concentrations of EDTA and EGTA could each be decreased to 5 mM without loss of the inhibitory effect. The disulfide-reducing agent DTT, at a concentration of 10 mM, revealed a slight interference with the process of penetration. Dansylcadaverine (1 mM) totally blocked this event, and a dose-response relationship was obtained when concentrations down to 0.1 mM were analyzed. No inhibition was seen when trifluoperazine, an antagonist to calmodulin, was present in the incubation medium. This was also the case for colchicine and cytochalasin B, agents interfering with micro-

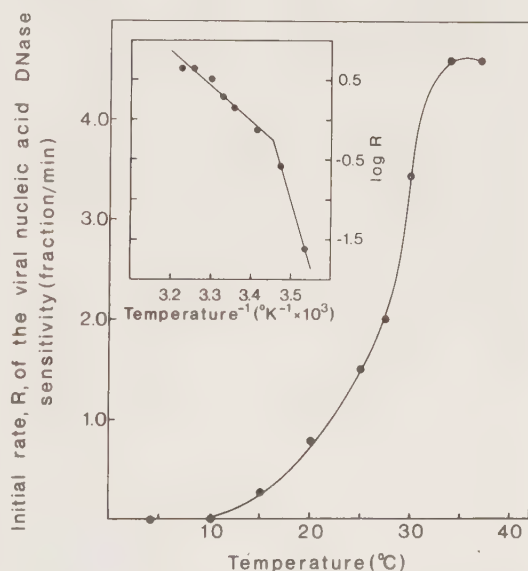


FIG. 2. Temperature dependence of the initial rate of adenoviral uncoating. The initial rates of viral uncoating at the different temperatures were calculated from the slopes in Fig. 1 to give the fraction of uncoated virions per minute. Insert. Conversion of the results into an Arrhenius plot.

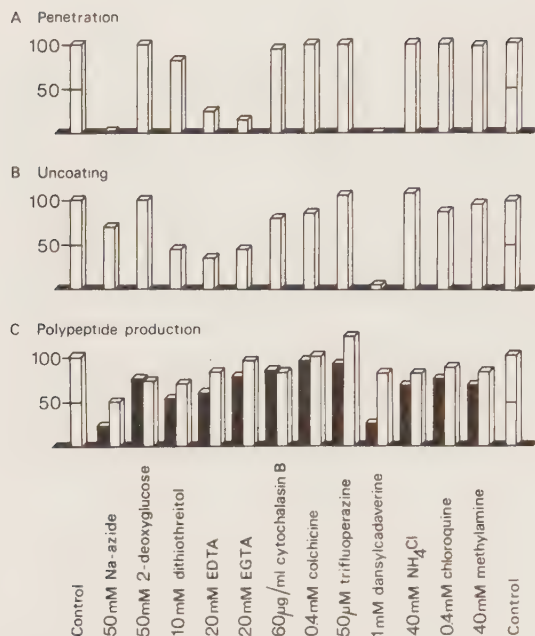


FIG. 3. Influence of added reagents on Ad2 internalization. (A) Penetration of Ad2 through the plasma membrane. 125 I-labeled antibodies against virus particles were used to follow the events of viral penetration. (B) Uncoating of Ad2, measured as the DNase sensitivity of the viral genome. (C) Synthesis of viral polypeptides. The amounts of DBP (solid bars) produced 7 h postinfection and of fiber antigen (empty bars) produced 13.5 h postinfection were determined by rocket immunoelectrophoresis. Ad2 was added to cells pretreated with the indicated reagents as described in the text. The control levels of the three processes, in normally infected cells, were set to 100%.

filaments and microtubules. None of the analyzed lysosomotropic agents, NH_4Cl , chloroquine, or methylamine, interfered with the step of internalization.

Uncoating in the presence of different reagents. The patterns of chemical interference with uncoating were similar to those obtained for penetration (Fig. 3A and B). One difference was the more pronounced inhibitory effect of DTT, which also displayed a dose-response relationship in the interval of 2 to 10 mM. Also noticeable was the poor effect of azide on the uncoating step, as compared with the significant inhibition of penetration. However, the combination of 50 mM azide and 50 mM 2-deoxyglucose resulted in a complete inhibition of the uncoating (data not shown). A dose-response relationship was obtained for dansylcadaverine at concentrations from 0.1 to 1.0 mM, whereas EDTA and EGTA showed almost the same inhibitory patterns even when the concentrations were decreased to 5 mM. An effect of ca. 20% inhibition was observed when cytochalasin B was present during the incubation of virus with cells.

Reversion of the inhibitory effect of different reagents on viral uncoating. Virus-infected cells were incubated in the presence of reagents, shifted to reagent-free media, and further incubated as described in the legend to Fig. 4. It was conclusively demonstrated that the inhibitions were re-

versed after the reagents were removed, even if these events proceeded slowly for some of the most potent inhibitors.

To determine whether it was possible to overcome the inhibition of uncoating caused by EDTA or EGTA, cells were carefully washed with 5 mM of either of these reagents before they were suspended in PBS containing 5 mM EDTA or EGTA. To each cell suspension was added 10 mM of Mn^{2+} , Mg^{2+} , or Ca^{2+} , and these mixtures were incubated at 37°C for 30 min. Virus addition and further treatments were as described above. It was reproducibly demonstrated that any of the analyzed bivalent cations could reverse the inhibitory effect of both EDTA and EGTA (data not shown).

Uncoating of Ad2 in cell-free extracts. Some of the results indicated that uncoating takes place at the plasma membrane. To attain more information, virus particles were attached to isolated plasma membranes or membranes of cell homogenate. The incubation mixtures were also supplemented with bivalent cations, energy donors in the form of

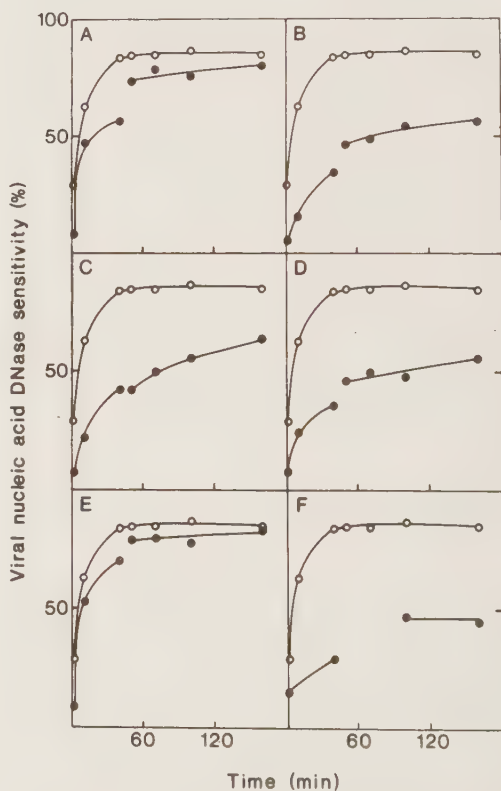


FIG. 4. Reversion of the inhibitory effect of different reagents on viral uncoating. Ad2 was attached for 5 min at 37°C to cells pretreated with indicated reagents. After removal of unattached virions, the infected cells were further incubated for 40 min at 37°C. At this point reagents were removed (gaps in the figures), and cell-virus mixtures were incubated for an additional period of 2 h. Virus uncoating was measured as described in the text. Panels illustrate the situation for (A) 50 mM sodium azide, (B) 10 mM DTT, (C) 20 mM EDTA, (D) 20 mM EGTA, (E) 60 µg of cytochalasin B per ml, and (F) 1 mM dansylcadaverine. Open circles indicate control series, and filled circles indicate reagent-treated series.

ATP or GTP. These incubations showed that none of the combinations analyzed possessed the capacity for a proper uncoating of adenovirus in vitro. This means that either the uncoating does not take place at the plasma membrane or that intact and functional cells are required.

Synthesis of virus-specified proteins. To monitor the indirect effects on gene expression of inhibitors of penetration and uncoating, we studied the production of an early viral antigen, DBP, and a late viral structural antigen, the fiber, in the presence of the previously described set of reagents. The inhibitory effects for most of the reagents were more pronounced for the synthesis of DBP at 6 to 8 h postinfection than for fiber synthesis at 12 to 15 h postinfection (Fig. 3C). It was demonstrated that the reagents which had a negative effect on penetration and uncoating also were efficient in blocking DBP synthesis. In addition, infections in the presence of lysosomotropic agents resulted in a 25 to 30% inhibition. The reagents which displayed a substantial impairment on the polypeptide production were tested at lower concentrations. Thus, 10 mM azide and 0.5 mM dansylcadaverine reduced the DBP production to 50% of the control values, whereas 5 mM DTT had no effect. Reagents which caused a distinctive and reproducible decrease in fiber production were azide, DTT, and 2-deoxyglucose. The lower interference of the majority of the reagents on fiber synthesis, compared with their effect on DBP production, was most probably due to the reversible nature of the inhibition when these reagents were removed, as was also demonstrated in the uncoating assay (Fig. 4). Supporting this idea was the finding that fiber production progressively increased up to control values during a 30-h kinetic study.

In a set of experiments reagents were present during the entire incubation period, to maintain the initial inhibitory effects on internalization. However, for most of the reagents the long exposure was lethal to the cells, as judged by trypan blue exclusion analyses.

Electron microscopy of early interactions between Ad2 and cells. To obtain further information regarding the effects of the reagents used, virus-infected cells were processed for electron microscopy. Virion localization, as revealed by direct visualization, corresponded well with previous data obtained by indirect techniques. Thus, the most potent inhibitors of penetration and uncoating yielded a large proportion of virus left on the cell surface or enclosed within vesicles in the cytoplasmic compartment. In the case of sodium azide, only 1% of the scored Ad2 particles were released as free entities inside the cytoplasm, which should be compared with 82% in the control series (Table 1 and Fig. 5). Also for dansylcadaverine, DTT, and EDTA, the amounts of free virus particles inside the cells were low, 11 to 14%. On the other hand, cells treated with these reagents or cytochalasin B showed relatively higher proportions of virions inside vesicles. It was observed that virions were internalized in vesicles, of which a predominant fraction appeared coated (Fig. 5B). Vesicles containing one virus particle were most frequent, except when cells were treated with DTT, in which case vesicles were noncoated (Fig. 5C).

DISCUSSION

To continue our previous studies on the specific and cooperative binding of Ad2 to HeLa cells, we have, in the present investigation, focused on the subsequent steps of virion internalization. In close connection to the penetration of the plasma membrane, it has been shown by others that the virion capsid is destabilized (24, 34, 38).

The uncoating kinetics of cell-attached Ad2 was strongly

temperature dependent, as previously indicated by Philipson (34) and Lawrence and Ginsberg (24). The maximum level of DNase sensitivity of the parental DNA was also in excellent correspondence with previous results (24, 34). Calculated figures of activation energies for the process of uncoating yielded values in correspondence to reports for membrane processes above and below lipid phase transitions (19) and for established systems of endocytosis (26). A similarly strong temperature dependence for the endocytotic uptake of Semliki Forest virus (27), asialoglycoprotein (40), and the plant toxin ricin (35) has also been reported.

To unravel the mechanisms behind adenovirus internalization, we analyzed the effect of twelve reagents on the steps of viral penetration, uncoating, and early and late gene expression. Under the conditions employed, none of the reagents were deleterious to cells. Our biochemical data were supported by visualization in the electron microscope of the actual cellular distribution of virions. All processes were energy dependent, since they were more or less inhibited by sodium azide. Interestingly, the penetration step was completely blocked, whereas uncoating was inhibited only by 25%, indicating a virion capsid labilization already at the outer surface of the plasma membrane and possibly in close connection with previously stated patching of the viral receptors (32, 33). This finding is analogous to the situation in the nonenveloped picornavirus system, which also begins with a modification of virions already at the cell surface (9). On the other hand, the more complex RNA-viruses of the *Reoviridae* have been reported to undergo a partial uncoating after entry into the cytoplasmic compartment (37). For another nonenveloped virus family, the *Papovaviridae*, studies in the simian virus 40 system have indicated that the nucleus is the major site for virion uncoating (18).

To further characterize the initial step of adenovirus destabilization, we analyzed the capacity of isolated plasma membrane fractions and cell homogenates to mediate this process, in the presence and absence of exogenously added energy donors and bivalent cations. Since the procedure for plasma membrane isolation has been reported to be critical for the labilization of picornaviruses in vitro (9), we employed the two-phase separation system. Our efforts to uncoat adenovirions in vitro turned out negative, thus supporting the previous data of Brown and Burlingham (3) but contradicting the findings of Boulanger and Warocquier (2). However, the important conclusion of these observations is

TABLE 1. Cellular distribution of Ad2 particles in response to different reagents as revealed by electron microscopy^a

Reagent	Relative amount of virus particles (%) ^b		
	On cell surface	Within vesicles	Free in cytoplasm ^c
Control	13	6	82 (65)
50 mM NaN ₃	84	15	1 (0)
50 mM 2-deoxyglucose	42	5	52 (45)
10 mM DTT	63	26	12 (17)
5 mM EDTA	67	19	14 (31)
60 µg of cytochalasin B per ml	57	19	24 (39)
0.4 mM colchicine	40	8	52 (27)
0.5 mM dansylcadaverine	67	20	12 (19)

^a Ad2 was added to cells pretreated with indicated reagents and incubated at 37°C for 20 min, after which cells were processed as described in the text.

^b Relative distribution of 250 counted virions per sample series.

^c Numbers in parentheses represent the percentages of free virus particles in close proximity to nucleopores.

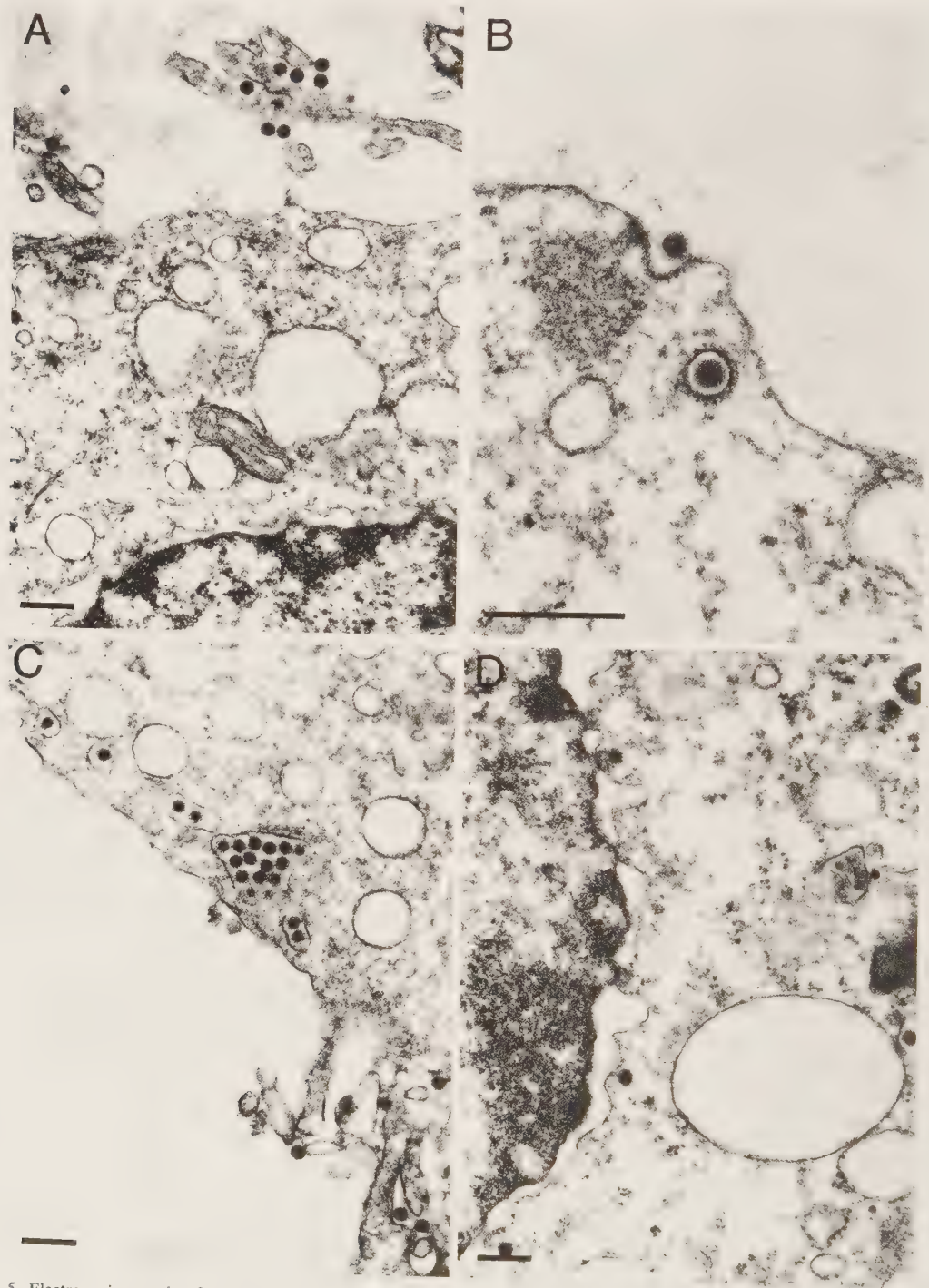


FIG. 5. Electron micrographs of Ad2-infected HeLa cells. Cellular distribution of virions 20 min after infection at 37°C. Panels show cells pretreated with (A) 50 mM sodium azide, (B) 50 mM 2-deoxyglucose, and (C) 10 mM DTT. (D) Control infection without added reagents. Bar, 300 nm.

that intact and metabolizing cells are needed to provide the exact microenvironment for adenovirus uncoating.

Regarding the penetration-uncoating system, a situation opposite to the effect of azide treatment was provoked in the presence of the disulfide-reducing agent DTT. Thus, penetration was close to normal, whereas uncoating was inhibited by ca. 50%. Interestingly, a fourfold increase over the control value of the frequency of virions remaining within cytoplasmic vesicles was observed in the presence of DTT. A possible explanation for this finding could be that internalized but somehow unmodified virions were unable to escape from endocytotic vesicles. It has been reported that DTT inhibits the clustering of enkephalin receptors (16). A corresponding effect can indeed be the case regarding the patching of adenovirus receptors, and this process may be a prerequisite for a proper uncoating.

The steps of penetration and uncoating were further shown to be dependent on the presence of bivalent cations. Since no inhibitory effects or only weak ones were revealed after the addition of trifluoperazine, colchicine, and cytochalasin B, we conclude that the demonstrated cation dependence was not connected with the function of calmodulin or elements of the cytoskeletal system. Dales and Chardonnet (7) showed for the Ad5 system, in the presence of colchicine, a close to normal transport of virions from the plasma membrane region to the nucleus, indicating that intact microtubules are not essential for this migration. This lack of drug effect is also supported by our data on gene expression.

Dansylcadaverine, an established inhibitor of liver and serum transglutaminases (8), has been shown to efficiently block the redistribution of receptor-ligand complexes and subsequent steps of receptor-mediated endocytosis, e.g., of vesicular stomatitis virus and α_2 -macroglobulin (31). In our study, the steps of virion penetration and uncoating were drastically inhibited by this reagent. Therefore, a preceding reorganization of attached virions and an involvement of a bivalent-cation-dependent transglutaminase activity are suggested for uncoating and the adsorptive endocytosis of Ad2.

For the well-characterized system of the enveloped Semliki Forest virus, endocytotic internalization and passage through acid vesicles have been demonstrated (27). In a previous study Boulanger et al. (1) put forward the idea that a lysosomal route might be important for adenovirus destabilization. However, attempts for in vitro uncoating of Ad2 and Ad5 in the presence of lysosomal extracts turned out negative (1). In our study none of the lysosomotropic agents displayed a significant effect on penetration or uncoating, indirectly indicating the lack of acid vesicles in the latter process. This finding is also corroborated by our observation that adenovirions become sensitive to DNase treatment already at the site of the plasma membrane. In the presence of lysosomotropic agents, the synthesis of an early viral antigen (DBP) was reduced by 25 to 30%, whereas the production of the late fiber antigen was inhibited by ca. 15%. These results and the reversible nature of inhibition indicate that virus particles might be transported through acidic vesicles before genome expression takes place in the nucleus. It has also been demonstrated by Ohkuma and Poole (30) and described for the Semliki Forest virus infection (17) that the effect of lysosomotropic reagents is rapidly reversed upon removal. To continue this investigation it would be of interest to analyze an event immediately following a presumptive viral passage through an acidic vesicle.

In the present investigation we have tried to survey the mechanisms of Ad2 internalization. From previous and present results we suggest the following flow of events. Ad2

virions are attached to specific receptors on the plasma membrane of HeLa cells (39). The receptors are subsequently redistributed in the membrane, leading to a clustering of attached virions (33). This reorganization mediates a destabilization of the virus particles leading to a DNase-sensitive viral genome. The modified virions are internalized via vesicles, probably coated, which subsequently develop into endosomes. Uncoating might be a prerequisite for virions to escape from these intracellular vesicles, on the route to the nucleus. During this transport of Ad2 from the cell surface to the nucleus, elements of the cytoskeleton do not seem to be involved. In summary, we interpret our data as favoring a model of adsorptive uptake of Ad2, which resembles the process of receptor-mediated endocytosis defined by Goldstein et al. (14).

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VIRUS-RECEPTOR INTERACTION IN THE ADENOVIRUS SYSTEM.

III. Characterization of the positive cooperative binding of virions on HeLa cells.

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ABSTRACT

The established positive cooperativity of adenovirus 2 binding to HeLa cells, revealed a strong temperature dependence. The degree of cooperativity, quantified by means of Hill coefficients, progressively increased from 10°C to reach a maximum level, which was maintained between 20 and 37°C. On the other hand, negative cooperativity of virion attachment was apparent at 3.0°C and on glutaraldehyde-stabilized cells. The corresponding monovalent ligand of the system, the fiber antigen, demonstrated only weak positive cooperativity of the binding at 37.0°C, which was absent at 3.0°C.

Dithiothreitol and dansylcadaverine, reagents inhibiting clustering of ligand-receptor complexes in the plasma membrane, markedly reduced the degree of positive cooperative binding at 37.0°C.

Evidently, the positive cooperative binding of adenovirus to HeLa cells at 37.0°C is a consequence of both the multivalency of virus attachment proteins, i.e. fibers, on the virion, and of the capacity of the receptor sites to migrate in the plane of the plasma membrane, forming local aggregates of virus-receptor site complexes.

INTRODUCTION

Recently, it has been suggested that adenovirus attachment to cells leads to rearrangements in the plasma membrane (10, 11, 20, 21), resulting in cooperative binding and capping of the virus particles (21). On HeLa or KB cells there are 5,000 to 10,000 specific binding sites (13, 23), which are referred to as cellular receptor sites (CRS). The fiber antigen, localized in the vertex regions of the virus particle, has been identified as the virus attachment protein, since it specifically prevents virion attachment after cells have been pretreated with this protein (23). The number of receptor sites for the fiber has been estimated to be one to two orders of magnitude over that for virions on HeLa or KB cells (13, 23).

The importance of lateral migration and clustering of ligand-receptor complexes, in the plasma membrane, has been demonstrated as an integral part of the mechanism leading to the cellular activation in response to peptide hormones (14, 27, 28). Furthermore, it has conclusively been shown by Prujansky et al. (25) that mitogenic stimulation of lymphocytes by lectins requires multivalent ligands. Positive cooperative binding is also apparent with such multivalent lectins, but not with the correspondingly monovalent ones. These authors put forward the idea that clustering of lectin-receptor complexes and conformational changes in membrane structure are prerequisites for mitogenic stimulation (25).

Positive cooperative binding of ligands could imply that binding of the first ligand to one receptor site promotes the binding of subsequent ligands to the other sites. On the other hand, negative cooperativity describes the situation when the binding of one ligand

impairs the attachment of the following ligands (for a review, see Neet (17)). The Hill coefficient (12), is a most convenient means to quantitatively express the degree of cooperativity, and this factor is determined from the slope of a corresponding Hill plot.

In this report attempts have been made to characterize the situation leading to positive cooperative binding of adenovirus 2 to HeLa cells, by comparing the attachment of virions to cells with that of the fiber antigen with respect to obtained Hill coefficients and binding affinities at 3.0 and 37.0°C. The effect of physiologically interacting reagents and the temperature dependence of the cooperative binding between 3.0 and 37.0°C was also studied.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenovirus 2 (Ad2) was propagated, radioisotope (^3H -thymidine) labeled, and purified as described previously (6).

Isolation and purification of the fiber antigen. Virus infected cells were disrupted and processed for virion isolation as previously reported (6). The virus-free supernatants, containing soluble cell and viral antigens, were subjected to anion-exchange chromatography as described by Pettersson et al. (22), with the following modifications: the buffer system of choice was 50 mM Tris-hydrochloride, pH 7.7, and the sodium chloride gradient ranged from 0 to 0.4 M. Fiber antigen containing fractions were collected after identification by double radial immunodiffusion (18) against monospecific anti-fiber antiserum. After extensive dialysis against 50 mM sodium phosphate buffer, pH 7.2, containing 0.2 M sodium chloride, the isolated fiber was applied on a column of wheat germ agglutinin-Sepharose 6MB (0.9x6.3 cm or 4 ml), equilibrated in the same buffer. After elution with 100 mg per ml of N-acetyl-D-glucose-amine, the purified fiber was dialyzed extensively against double-distilled water and subsequently freeze-dried.

Radioisotope labeling of the fiber antigen. The purified fiber, dissolved in 0.1 M sodium borate buffer, pH 8.1, containing 0.15 M sodium chloride, was ^{125}I -labeled with 1,3,4,6-tetrachloro-3a,6a-diphenylglucoluril (Iodogen) as described by Fraker and Speck (7). The specific radioactivity of the labeled fiber was 2.9×10^8 cpm per mg of protein.

Attachment studies. The general procedure of virion attachment was as outlined earlier (31). HeLa cells were washed once in phosphate-buffered saline (PBS) and suspended to 5×10^7 cells per ml, and in all experiments, cells were equilibrated at the appropriate temperature for 10 min before the addition of the ligand. ^3H -thymidine labeled virions (1.0×10^7 to 1.5×10^7 cpm per 10^{12} virions) were allowed to attach to cells at multiplicity of infections (MOIs) between 50 and 20,000. Virus-cell mixtures were incubated for 45 min at 37.0°C or 180 min at 3.0°C . These periods were selected to ensure saturated binding at equilibrium (21). In temperature experiments, the virion attachment was assessed between 3.0 and 37.0°C , after selected incubation periods according to above.

Attachment of virus particles at 37.0°C was also measured in the presence of the following reagents: 1) Sodium azide (50 mM), 2) 2-deoxyglucose (50 mM), 3) dithiothreitol (DTT, 5 mM and 10 mM), 4) EDTA (5 mM and 20 mM), 5) ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 5 mM and 20 mM), 6) cytochalasin B (60 $\mu\text{g}/\text{ml}$), 7) colchicine (0.4 mM), 8) trifluoperazine (50 μM), 9) dansylcadaverine (0.5 mM and 1.0 mM), 10) ammonium chloride (40 mM), 11) chloroquine (0.4 mM), and 12) methylamine (40 mM). The cells were preincubated with the appropriate reagent thirty min before virus addition.

Attachment of ^{125}I -labeled fiber antigen was assessed in analogy with virion attachment as outlined above. Ratios of fiber molecules over cell numbers were between 2,000 and 400,000, and incubation periods for cells and added fiber antigen were 30 and 120 min at 37.0 and 3.0°C , respectively. For calculations a 180,000 dalton molecular weight of the native fiber antigen was used (4, 30).

Scatchard and Hill plots. Graphical representations according to Scatchard (26) were made for binding data obtained for virions and fibers. For each graph a corresponding Hill plot (12) was constructed in which $\log B/n-B$ was plotted against $\log F$; B and F represent bound and free ligands, respectively, and n is the actual number of receptor sites per cell for each series, derived from the intercept on the abscissa of the Scatchard plot.

Protein determination. Protein was determined by the method of Hartree (8) with bovine serum albumin as the standard.

Liquid scintillation spectrometry. Direct radioactivity measurements of water-containing samples were made in Ready-Solv HP/b. A Mark II scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) was used to determine radioactivity.

Chemicals and reagents. Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories Ltd, Irvine, Scotland. ^3H -thymidine (75 Ci/mmol) was purchased from New England Nuclear Chemicals GmbH, Dreieich, Federal Republic of Germany and the ^{125}I -iodine (13-17 mCi/ μg) from Amersham International plc, Buckinghamshire, England. Bovine serum albumin, chloroquine, colchicine, cytochalasin B, DTT, EGTA, monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemicals Co., St. Louis, Mo. Ammonium chloride, 2-deoxyglucose, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic of Germany and N-acetyl-D-glucosamine was from BDH, Chemicals Ltd, Poole, England. Wheat germ agglutinin-Sepharose 6MB was obtained from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril

(Iodogen) was from Pierce Chemicals Co., Rockford, Ill, and the Ready-Solv PH/b was purchased from Beckman Instruments AB, Bromma Sweden.

RESULTS

Virion attachment. Ad2 was added to HeLa cells at different MOIs ranging from 50 to 20,000. To ensure proper equilibrium binding at 37.0 and 3.0°C, virus-cell mixtures were incubated for 45 and 180 min, respectively. Fifteen separate binding experiments with 12 measuring points for each series were performed at 37.0°C. A typical Scatchard plot for virion binding at this temperature revealed a maximum of the curve indicating positive cooperativity (Fig. 1A). The average number of CRSs was estimated to be between 3,700 and 5,900 and a binding constant per site between 1.0×10^{10} and $2.2 \times 10^{10} \text{ M}^{-1}$ was obtained.

Graphical representation of virion binding data at 3.0°C according to Scatchard, revealed an upward curvature, indicative of negative cooperativity (Fig. 1A).

To obtain quantitative, comparable and more easily interpretable views of the Scatchard plots, the previous results, and binding data to follow, were converted into Hill plots (Fig. 1B). Hill coefficients were calculated from the slopes of the plots, and coefficients ranging from 1.35 to 1.47 and 0.94 to 0.96 were obtained for virion binding at 37.0 and 3.0°C, respectively. It should be pointed out that all Hill coefficients in this report were based on the first six measuring points representing the low and medium multiplicity range, including the maximum regions of the corresponding Scatchard curves for all 37.0°C series. A value above 1.0 for the slope of a Hill plot indicates a positive cooperativity for the observed ligand-receptor interaction.

For the low temperature series, the computer program "LIGAND" developed by Munson and Rodbard (16) was used to fit the experimental

data. A two receptor site model with different affinities emerged as a result of the calculations. Figure 2 shows the Scatchard plot for the low temperature series of Fig. 1A, with the addition of the computerized lines I and II depicting the suggested two component receptor system. Obtained intrinsic binding constants of 1.5×10^{10} and $7 \times 10^8 \text{ M}^{-1}$ for the high (I) and low (II) affinity sites, respectively, are represented by the lines. Calculated receptor numbers for the high and low affinity sites were 1,200 and 5,500, respectively. The horizontal line III is the computer representation for nonspecific binding.

Attachment of the fiber antigen. Equilibrium binding studies of affinity purified fiber antigen to cells, were performed as described in Materials and Methods. Scatchard plots of the fiber attachment, yielded almost straight lines at 3.0°C (Fig. 3A). From corresponding Hill plots, Hill coefficients of 0.99 to 1.03 were obtained, thus strengthening the idea of non-cooperative binding indicated in the Scatchard plot (Fig. 3B). The binding at 37.0°C showed slight down-ward curves in the Scatchard analyses and yielded Hill coefficients between 1.05 and 1.15, suggesting a weak positive cooperative binding at this temperature. The estimated number of binding sites at both temperatures ranged between 85,000 and 95,000 and calculations of the association constants yielded values from 1.0×10^8 to $2.7 \times 10^8 \text{ M}^{-1}$. Attachment of fiber antigen, metabolically labeled with ^{35}S -methionine (5), displayed under identical conditions almost the same binding pattern as the ^{125}I -labeled fiber, with an estimated association constant of $2.9 \times 10^8 \text{ M}^{-1}$ and a number of binding sites of around 110,000 (not shown).

The cooperative binding of Ad2 at different temperatures. As graphically demonstrated by plotting calculated Hill coefficients against various attachment temperatures, the positive cooperative binding of virions exhibited strong temperature dependence, with a two-critical temperature appearance (Fig. 4). At temperatures below 6°C, the Hill coefficient displayed a lowest value of 0.95, while a shift occurred at 7 to 8°C revealing a plateau with a coefficient of around 1.08. At increasing temperatures up to 20°C, the Hill coefficient progressively increased to finally reach a maximum level of 1.42, which was maintained in the interval of 20 and 37°C.

The effect of different reagents on the cooperative binding of Ad2 at 37°C. The most pronounced inhibitory effect on the positive cooperative Ad2 binding was obtained by dithiothreitol at a concentration of 10 mM, followed by the effect of 1.0 mM dansylcadaverine (Fig. 5). All the other reagents investigated were noneffective. Thus, the positive cooperative binding was not dependent of accumulated energy in the form of ATP, nor appeared the cytoskeleton system to be involved. Neither EDTA, EGTA, trifluoperazine nor the lysosomotropic agents, at indicated concentrations, affected the positive cooperativity of Ad2 binding to HeLa cells.

Attachment of virions at 37.0°C to HeLa cells stabilized with 0.015% glutaraldehyde (21), was comparable to the situation at 3.0°C with an estimated Hill coefficient of 0.99.

DISCUSSION

The binding mechanisms of Ad2 virions and fiber antigen to HeLa cells were studied at equilibrium, i. e. after incubation periods long enough to achieve saturated binding (29,33). It is of greatest importance, in binding studies of ligands to cells, to be certain that obtained results are reflections of true attachment, and not include effects of e. g. internalization of ligand-receptor complexes or modifications of the ligand as discussed by Beck and Goren (1). Ways to overcome this problem is to decrease the temperature and thereby prevent internalization, or to add reagents inhibiting such side-reactions without affecting the apparent attachment.

It was reproducibly shown that virion attachment at 37.0°C exhibited positive cooperativity, with an estimated mean Hill coefficient of 1.41. From the Scatchard plots the determined number of CRSs were between 3,700 and 5,900 and the calculated association constants ranged between 1.0×10^{10} and 2.2×10^{10} (M^{-1}), as previously proposed (21). The binding of the fiber antigen displayed non-cooperativity at 3.0°C, whereas a tendency of positive cooperative binding at 37.0°C was observed. Wadell and Norrby (34) have reported that a small fraction of the fiber antigen, from adenovirus belonging to subgroup C, and isolated by ion-exchange chromatography, has the ability to dimerize. Such bivalent structures might bind to cells in a cooperative way, yielding the increased mean Hill coefficient of 1.10 at 37.0°C as compared with 1.01 obtained at 3.0°C. The mean number of receptor sites for the fiber antigen on HeLa cells was estimated to 90,000 with a mean association constant of 2.3×10^8 (M^{-1}), at both 37.0 and 3.0°C. This is in fairly good agreement with previously reported data on attachment of fibers to isolated KB cell plasma membranes (13) and also with the

calculated number of fiber receptors on HeLa and KB cells (23). The possibility that the in vitro-labeled fiber antigen could yield erroneous binding data due to chemical modification(s) (2), was in the presented study not considered, since in vivo-labeled fiber exhibited almost identical attachment characteristics as the iodinated antigen. Attachment of virions at 3.0°C showed an upward curve in the Scatchard plot, and a Hill coefficient slightly below one (i. e. 0.95) was obtained, indicating negative cooperative binding. The engagement of high and low affinity sites was also revealed after computerized model fitting of the experimental data. Even though the low number of measuring points, especially at high MOIs, made the calculations not statistically significant, it is reasonable to believe that attachment of virions at 3.0°C, and low MOIs, yield an association to cells with the same binding affinity as at 37.0°C. On the other hand, at high MOIs, the competition for receptor sites will cause virions to bind monovalently, with respect to the fiber antigen, and with an affinity in the range of that of isolated fiber.

The positive cooperativity was shown to be highly temperature dependent, with two shifts in the temperature regions at 7 to 8°C and 19 to 20°C. We interpret the shifts in the temperature dependent positive cooperativity in terms of local phase transitional/separational effects on the receptor- ligand domains of the plasma membrane. A likewise change in the physical status of the lipid matrix, has been suggested for the temperature dependent uncoating of adenovirions associated with the plasma membrane surface (32). Correspondingly local thermal transitions have been proposed as an explanation for the temperature dependent intercellular adhesiveness of HeLa cells (3), in spite of failure in establishing temperature dependent shifts in membrane microviscosity.

These studies of changes in microviscosity were performed by fluorescence polarization of incorporated 1,6-diphenyl-1,3,5-hexatriene in the bulk of the cell surface lipids. If one wishes to study the interactions of receptor-ligand complexes within the lipid matrix, the annular lipids comprising the immediate environment are those of interest. Therefore, the most relevant means of studying the migrational behaviour of viruses attached to cellular receptor sites in the plasma membrane, might be the employment of rhodamine labeled virions (21) in connection with the FRAP (fluorescence recovery after photobleaching)-technique, as introduced by Poo and Cone (24) and Liebman and Entine (15).

Different reagents, previously employed to manipulate the steps connected with internalization of Ad2 virions (32), were used to further characterize the positive cooperative binding of Ad2 at 37.0°C. The presence of dithiothreitol and dansylcadaverine markedly reduced the degree of positive cooperativity of the binding. These reagents have been shown to efficiently block the clustering of receptor-ligand complexes in the membrane (9,19). The results above together with the facts that attachment of virions at 37.0°C to glutaraldehyde-fixed cells exhibited no positive cooperativity (Fig. 5), and the negative cooperative binding at 3.0°C (Fig. 1 and 5), all strongly suggest that aggregation in the plasma membrane of virion-receptor site complexes is a prerequisite for the achievement of positive cooperative binding of Ad2 virions. The addition of sodium azide, a potent inhibitor of Ad2 internalization (32), or the reagents interfering with the cytoskeletal elements, displayed no effect on the positive cooperativity. This implies that the aggregation in the membrane, is a relatively local phenomenon and distinct from the previously reported capping of bound virions (21).

From the presented results of the Ad2 virion and fiber attachment to HeLa cells, we propose that the unequivocal positive cooperativity of virion attachment at 37.0°C is a function of *both* the multivalency of virion attachment proteins on the actual virus particles, and the ability of the receptor sites to move laterally in the plasma membrane, forming local aggregates of virus-receptor site complexes.

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FIGURE LEGENDS

Figure 1

Equilibrium binding of adenovirus to HeLa cells. Ad2 particles were added to cells at different MOIs and the number of bound virions was estimated after 45 min at 37.0°C (solid circles) and 180 min at 3.0°C (open circles). Data from a typical experiment was converted into a Scatchard plot (A) and a Hill plot (B). N_H ; Hill coefficient.

Figure 2

Scatchard plot of adenovirus binding to HeLa cells at 3.0°C. Data from Fig. 1, was analyzed by the "Ligand" computer program. Lines I and II represent the obtained virion binding to high and low affinity sites, respectively. Line III equals the nonspecific binding of the viruses.

Figure 3

Equilibrium binding of fiber antigen to HeLa cells. Fiber was added to cells at ratios between 2,000 and 400,000 molecules per cell. The amount of bound fiber was measured after 30 min at 37.0°C and 120 min at 3.0°C (solid and open circles, respectively). Data represent a typical experiment. A) Scatchard plot. B) Hill plot. N_H ; Hill coefficient.

Figure 4

Temperature dependence of the cooperative binding of adenovirions to HeLa cells. Different MOIs of Ad2 particles were added to cells, equilibrated at different temperatures. The attachment was measured as equilibrium binding at appropriate temperatures, and for each temperature a separate Hill plot was constructed, from which Hill coefficients (N_H) were derived.

Figure 5

Influence of different reagents on the positive cooperative binding of adenovirus. Cells were preincubated at 37.0°C with the appropriate reagent for 30 min before virus addition. After 45 min of virus-cell interaction, the number of bound virions was estimated, and Hill coefficients (N_H) were calculated.

* cells were fixed with 0.015% glutaraldehyde (21).

** cells treated with 20 mM of either EDTA or EGTA, had a tendency to aggregate.

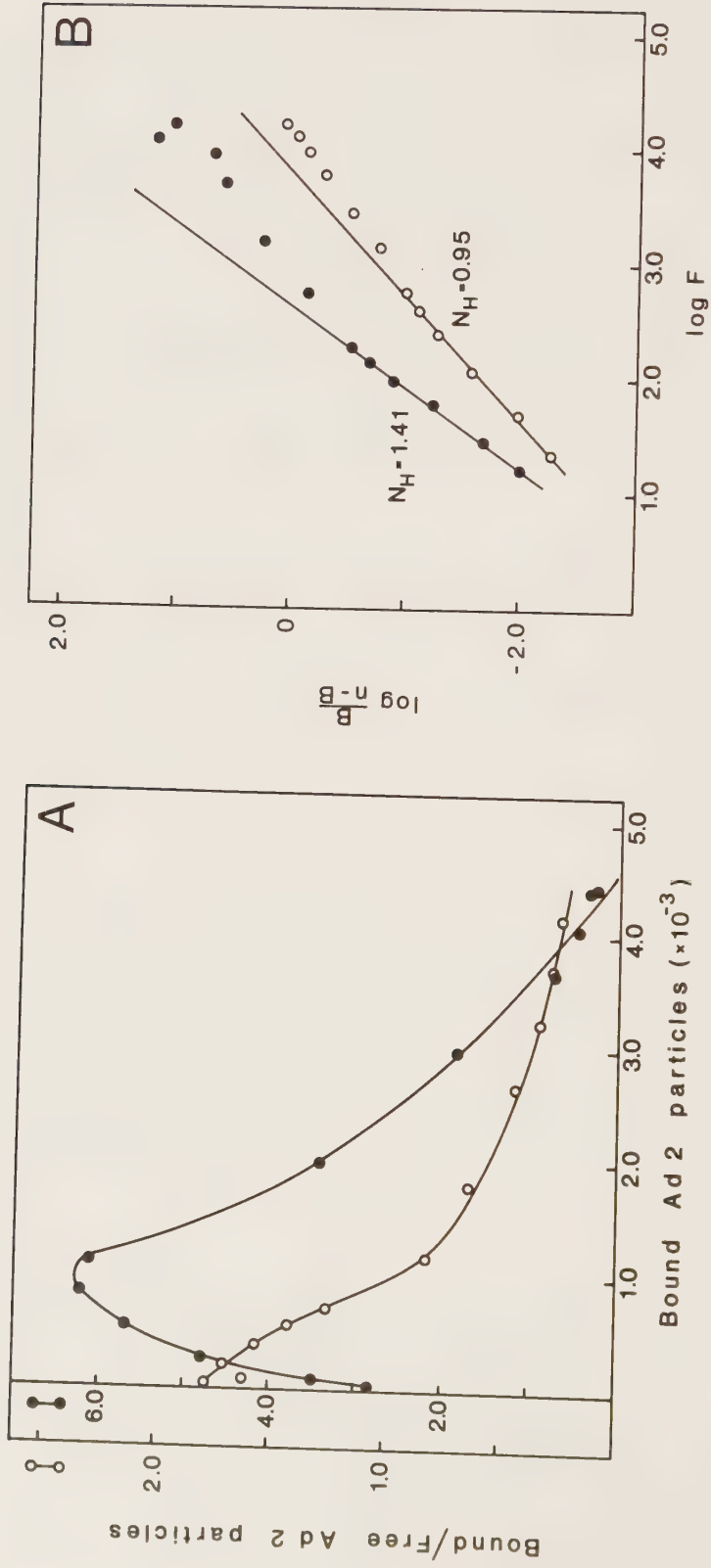


Figure 1A and B.

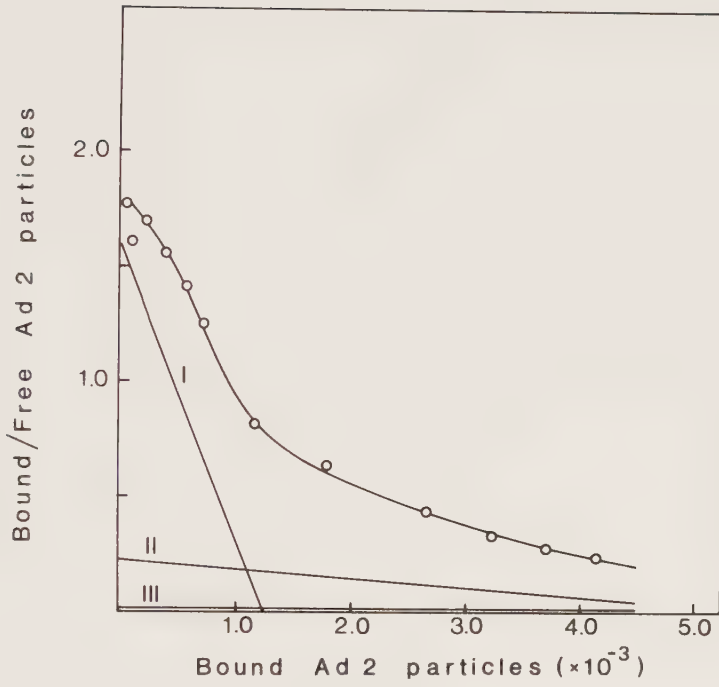


Figure 2.

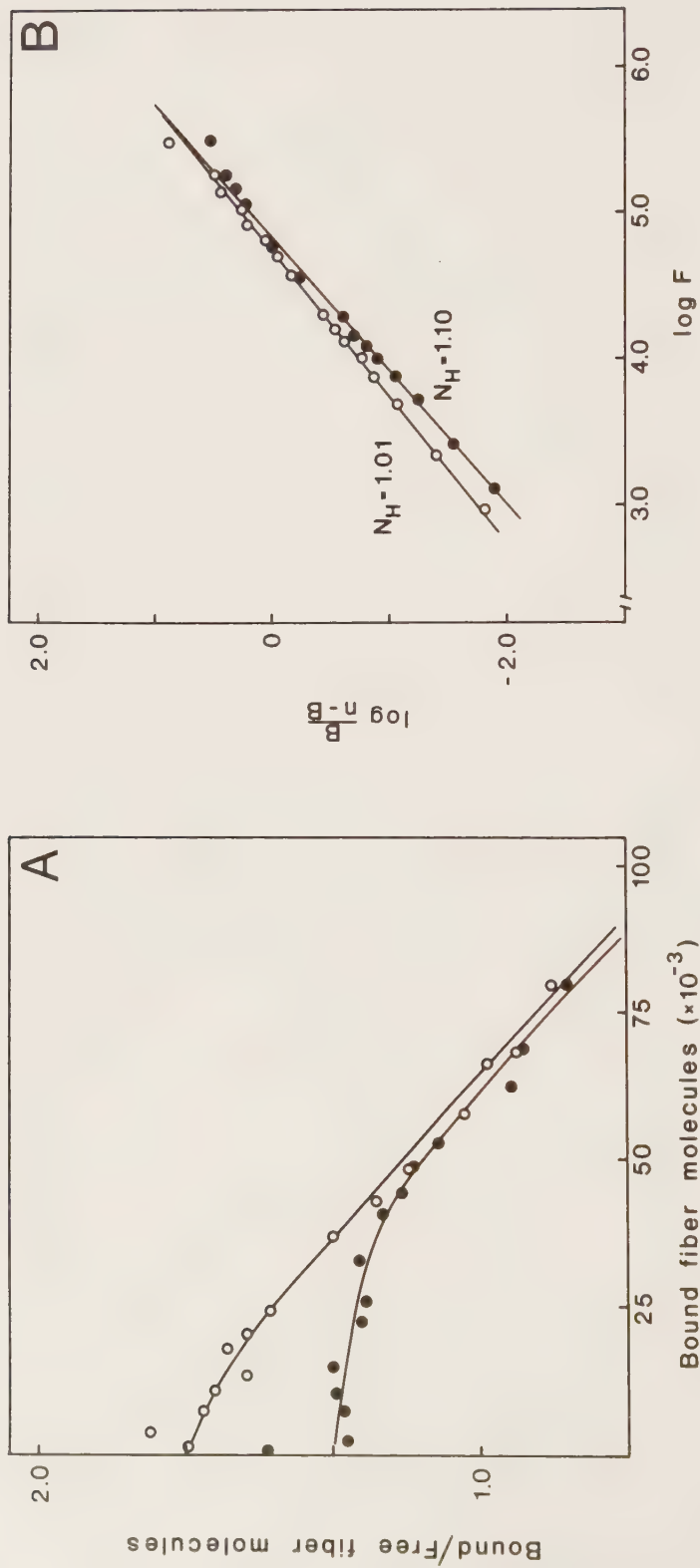


Figure 3A and B.

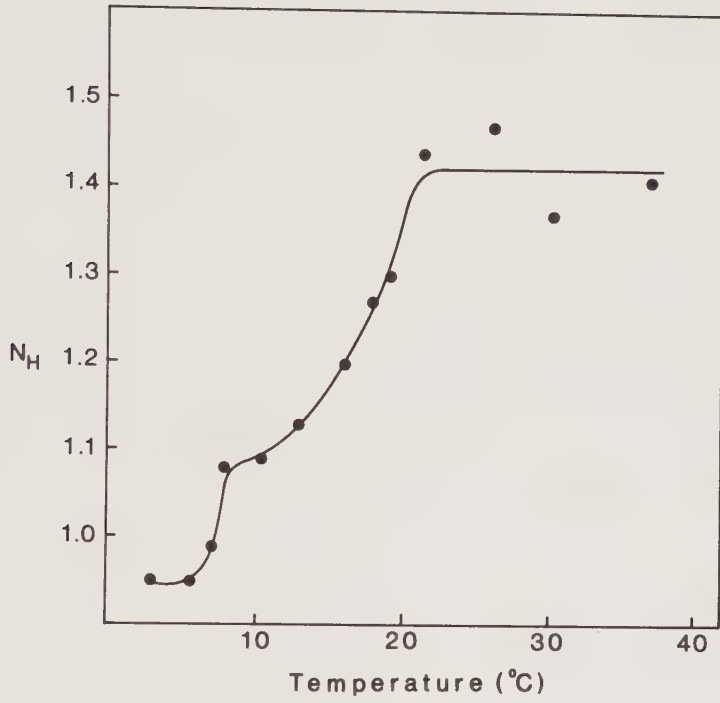


Figure 4.

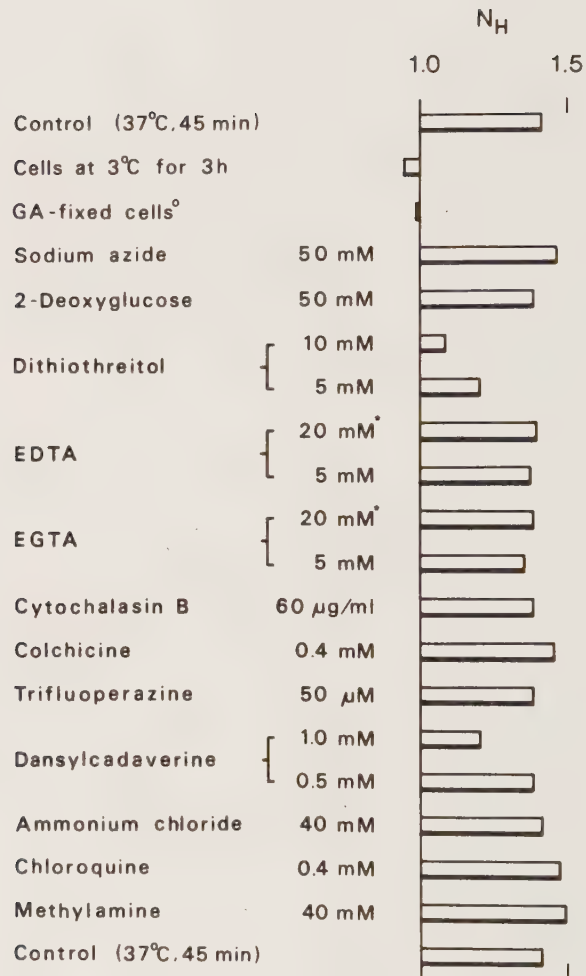


Figure 5.

v

VIRUS-RECEPTOR INTERACTION IN THE ADENOVIRUS SYSTEM.

IV. Immunochemical identification of a cellular
receptor protein.

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ABSTRACT

Treatment of HeLa cells with an antiserum against total plasma membranes, inhibited subsequent adenovirus 2 attachment. The degree of inhibition was highly dependent on the cell concentration and temperature during the antiserum treatment. Crossed immunoelectrophoretic analyses of Triton X-100-solubilized membrane proteins, with this antiserum, reproducibly revealed a reference pattern of 20 immunoprecipitates. To identify components of the cellular receptor sites for adenovirus, in the pattern of immunoprecipitates, intermediate agarose gels containing different ligands immobilized to Sepharose 4B were used. One precipitate of the reference pattern diminished when the fiber antigen, the previously established virus attachment protein, was the ligand of the intermediate gel. The major structural protein of the virus particles, the hexon antigen, did not display any effect. The use of immobilized wheat germ agglutinin in the intermediate gel had a drastic effect on the immunoprecipitation pattern. Thus, one third of the precipitates was reduced in height, including the precipitate specifically affected by the presence of the fiber antigen. Selectively cut out immunoprecipitates from crossed immunoelectropherograms, were further used as immunogens, to obtain antisera with higher relative specificity against the cellular receptor sites. One antiserum recovered from such immunizations, strongly inhibited the viral attachment. Moreover, this antiserum contained antibodies directed against the membrane component, which was affected by the fiber antigen. Immunoprecipitations in solution of ^{125}I -labeled membrane proteins with this antiserum, specifically revealed two polypeptides with the molecular weight of 39,000 and 51,000.

INTRODUCTION

At infection adenovirus 2 and 5 attach to specific sites on the plasma membrane of HeLa and KB cells (16,26,28). Membrane components, with affinity for complete virions or structural antigens of the viruses (i.e. fiber and penton structures), have been identified from KB (15,22) and HeLa cells (32). From these studies, it has been suggested that the cellular receptor sites for adenovirus (19), constitute polypeptides with the apparent molecular weights of 78K, 42K, and 34K on KB cells (15,22), and 40 to 42K on HeLa cells (32). It has further been proposed that some of the polypeptides are glycosylated (22,32).

In order to characterize a given protein, most available techniques require the protein in a rather pure form. In contrast, crossed immunoelectrophoretic analyses can be performed with complex protein mixtures (18). This fact is especially relevant for membrane proteins, because of difficulties in their isolation. Furthermore, antibodies against proteins present in a membrane are rather easily obtained, since most, if not all, membrane proteins are immunogenic (5). Various membrane systems from eucaryotes and procaryotes have been characterized by means of immunoelectrophoretic analyses (for a review, see Bjerrum, (5)).

A preliminary immunochemical characterization of the cellular receptor sites for adenovirus 2 on HeLa cells is described in this report. An antiserum against isolated plasma membranes, which was shown to inhibit virus attachment, was used in crossed immunoelectrophoretic analyses of Triton X-100-solubilized membrane proteins. Selected immunoprecipitates from such analyses were further used as immunogens in rabbits, to obtain antisera with higher relative

specificity against the cellular receptor sites. To identify the cellular receptor proteins, the latter antisera were used to immunoprecipitate iodinated membrane proteins in solution.

MATERIALS AND METHODS

Cells and viruses

HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium, supplemented with 5% fetal calf serum and 5 μ g of gentamicin per ml. Adenovirus type 2 (Ad2) was propagated, radioisotope (^3H -thymidine) labeled, and purified as described previously (11).

Production of antiserum against plasma membranes

Plasma membranes from HeLa cells were prepared according to Brunette and Till (7), by a procedure as earlier modified (32). Intramuscular injections into rabbits were performed every third week during a period of 17 months. Prior to the immunization sequence each rabbit was bled and preimmune serum was collected. Each immunization sample consisted of 1.0 mg of total plasma membrane proteins in 300 μ l, which was vigorously mixed with an equal volume of Freund's adjuvant. For the first five months Freund's incomplete adjuvant was used, after which the complete adjuvant was employed. After a total period of 13 months of repeated immunizations, the rabbits developed ulcerations at the injection points and therefore the complete adjuvant was replaced by the incomplete ditto. The rabbits were bled every 2 to 6 weeks and the inhibitory effect on Ad2 attachment (see below) was assessed for each bleeding, whereafter the material was pooled and stored at -17°C .

Assay of the effect of anti-membrane antisera on the viral attachment

HeLa cells were washed once in phosphate-buffered saline (PBS) and suspended at concentrations between 1×10^6 and 5×10^7 cells per ml. Antisera or preimmune sera, heated at 56°C for 30 min to inactivate complement, were added to the cells at different dilutions, and incubated at 37 or 4°C for 60 min. The treated cells were subsequently washed twice with $500\ \mu\text{l}$ PBS and suspended to give 5×10^7 cells per ml. ^3H -thymidine labeled Ad2 particles at a multiplicity of infection (MOI) of 340, were allowed to attach to the cells for 45 min at 37°C . The virus-cell mixtures were diluted five times with PBS and the cells were pelleted. Attachment of virions was determined from the radioactivity of the pellets and the supernatants. Inhibiting capacities of antisera on the viral attachment were calculated according to:

$$\frac{\text{Adsorbed Ad2}(\%)_0 - \text{Adsorbed Ad2}(\%)_{\text{immun.}}}{\text{Adsorbed Ad2}(\%)_0},$$

where adsorbed $\text{Ad2}(\%)_0$ is the attachment of virions to cells treated with preimmune serum, and adsorbed $\text{Ad2}(\%)_{\text{immun.}}$ is the attachment to cells treated with antisera, obtained in response to total plasma membranes or isolated immunoprecipitates.

Purification and immobilization of viral fiber and hexon antigen

Virus-infected cells were ^{35}S -methionine labeled as described by Edvardsson et al. (10), and were disrupted and processed for virion isolation as previously reported (11). The supernatant containing soluble viral and cellular antigens, and freed of virions, was subjected to anion-exchange chromatography according to Pettersson

et al. (27) and Persson R., C. Wohlfart, U. Svensson, and E. Everitt (submitted for publication). Fractions containing fiber and hexon antigen were collected, after identification by double radial immunodiffusion against monospecific antisera (24). The fiber antigen was further affinity-purified on wheat germ agglutinin-Sepharose 6MB as described earlier (Persson R., C. Wohlfart, U. Svensson, and E. Everitt, submitted for publication). The specific radioactivity of the fiber was 7.9×10^6 cpm per mg. Fractions containing the hexon were dialyzed extensively against PBS and were applied on a column of Sepharose CL-6B (1.5 by 85.0 cm; 150 ml) equilibrated in PBS. The hexon was eluted with PBS at a flow rate of 6.8 ml per hour with 1.7 ml per fraction. The specific radioactivity of the purified hexon was 3.8×10^6 cpm per mg.

The two viral antigens and commercially purchased wheat germ agglutinin (WGA) were immobilized to cyanogen bromide-activated Sepharose 4B (20). The amount of immobilized fiber, hexon and WGA was 0.42, 0.56 and 4.2 mg per ml of Sepharose 4B, respectively.

Treatment of HeLa cells with purified fiber and hexon antigen

To HeLa cells washed once in PBS, and suspended at a density of 5×10^7 cells per ml, were added different amounts of purified fiber and hexon antigen and the samples were incubated at 30°C for 60 min. The antigen-cell mixtures were washed three times with PBS and the extent of adsorption of the viral antigens was determined from the radioactivity cosedimenting with the cells. ^3H -thymidine labeled virions were allowed to attach to antigen-treated cells at a MOI of 2,000 for 60 min at 37°C . Samples were withdrawn 0.5, 10, 20, 30,

45, and 60 min after the viral addition, diluted five times in PBS and the cells were subsequently pelleted. Virus attachment was determined from the radioactivity of the recovered supernatants.

Crossed immunoelectrophoresis of Triton X-100-solubilized plasma membrane proteins

Plasma membrane proteins, 5 mg per ml, were solubilized in the presence of 1% Triton X-100 (TX100) as reported earlier (32). The membrane proteins were analyzed by crossed immunoelectrophoresis against appropriate antisera, in 1.0% (w/v) agarose gels containing 0.5% TX100 essentially as described by Laurell (18). The buffer system was 73 mM Tris-24 mM barbitone, pH 8.6, supplemented with 0.45 mM calcium lactate. The first dimension electrophoresis was performed at 200 V for 60 min at 12°C, and the second dimension was electrophoresed at 75 V over night at 12°C.

In some analyses, intermediate agarose gels (2,31) containing either immobilized viral antigens or WGA were positioned between the gel strip from the first dimension and the antiserum-containing gels of the second dimension.

After the electrophoreses, the agarose plates were dried under pressure and were subsequently stained for proteins with 0.2% Coomassie brilliant blue R in methanol, acetic acid and double-distilled water (5:1:5). In some cases, the separation of membrane proteins in the first dimension was visualized after precipitation of the proteins with picric acid of 80% relative saturation in 3.5 M acetic acid. The denatured proteins were subsequently stained as described above.

Production of antisera against individual membrane proteins by introduction of agarose gel-containing precipitates into rabbits (29,33)

Thirty-six identical crossed immunoelectrophoretic analyses of 100 μ g of TX100-solubilized membrane proteins were performed as described above. Appropriate precipitates (designated I and II) from each plate were cut out and collected without prior staining, and were washed with 0.15 M sodium chloride. Before the immunization, the precipitates were sonicated four times for 30 sec on ice, and divided into four equal portions, which were stored at -17°C . Equal volumes of sonicated precipitates and Freund's complete adjuvant were mixed vigorously and injected intramuscularly into rabbits, according to the immunization scheme of Harboe and Ingild (13). After the fourth immunization the rabbits were boosted with total plasma membranes in Freund's complete adjuvant, and they were finally bled 10 days afterwards. Preimmunization serum was collected from each animal.

The recovered antisera against the two different precipitates were analyzed for their capacity to inhibit virus adsorption and were furthermore analyzed ^{by/}crossed immunoelectrophoresis as described above.

Iodination of TX100-solubilized membrane proteins and immunoprecipitations

TX100-solubilized membrane proteins were iodinated with the Bolton-Hunter reagent as described by the manufacturer. The specific radioactivity of the membrane proteins was 5.5×10^8 cpm per mg. The immunoglobulin fraction of the antisera raised against the individual precipitates (I and II) was salted out with 33% (w/v) ammonium sulfate. The precipitates were washed once with 40% (w/v) ammonium

sulfate and suspended in PBS. The suspended fractions were reprecipitated with 30% (w/v) ammonium sulfate, whereafter the precipitates were dissolved in PBS and desalted by passage through PD-10 columns, containing Sephadex G-25M equilibrated in PBS. To each of the immunoglobulin fractions PBS was added so as to obtain the same concentration of immunoglobulin as that found in the original unfractionated sera. Sodium azide was added to a final concentration of 0.2%. Immunoprecipitations of the ^{125}I -labeled membrane proteins with the different immunoglobulin fractions were performed essentially as reported by Kessler (17) and Persson et al. (25). Five or 25 μl of the immunoglobulin solutions were added to 67 μg of ^{125}I -labeled membrane proteins in 300 μl and the mixtures were incubated at 23°C for 60 min, and then for another 60 min at 0°C . Fifty or 250 μl of a 20% (w/v) suspension of heat-inactivated and formaldehyde-fixed *Staphylococcus aureus* (Cowan I) cells were added to each of the mixtures of labeled proteins and immunoglobulins, and these were incubated at 0°C for 20 min. After the latter incubation, the cells were pelleted and washed twice with a 50 mM Tris-hydrochloride buffer, pH 7.4, containing 0.15 M sodium chloride, 5 mM EDTA and 0.02% sodium azide. The bacterial cells with adsorbed immunocomplexes were finally suspended in 100 μl of a Tris-buffered disruption solution, pH=7.3, containing 2% sodium dodecyl sulfate (SDS), 5% β -mercaptoethanol and 8.7% glycerol. The suspensions were mixed vigorously and heated at 100°C for 3 min. After sedimentation of the cells, the supernatants were analyzed electrophoretically on SDS-polyacrylamide slab gels.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE analyses were performed on gel slabs (1.5 by 70 by 70 mm) in a linear gradient of polyacrylamide between 6 and 20% (w/v), essentially as described by Maizel (21). The ratio of acrylamide and bis-acrylamide was (37.5:1.0). After electrophoresis the gels were stained for proteins in 0.05% Coomassie brilliant blue R in methanol, acetic acid and double-distilled water (3:1:6). The gels were subsequently dried under vacuum at 60°C for 3 h. For autoradiography, gels were exposed to Kodak XG-1 X-ray films for 2 to 3 weeks.

Assay for protein

Protein was determined by the method of Hartree (14) with bovine serum albumin as the standard.

Liquid scintillation spectrometry

Direct radioactivity measurements of water-containing samples were done in Ready-Solv HP/b. A Mark II scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.) was used to determine radioactivity.

Chemicals

Eagle minimal essential medium, L-glutamine, gentamicin, and fetal calf serum were obtained from Flow Laboratories Ltd., Irvine, Scotland. Bolton-Hunter reagent (^{125}I ; 2,000 Ci/mmol) and ^{35}S -methionine (1,070 Ci/mmol) were purchased from Amersham International

plc, Buckinghamshire, England. ^3H -thymidine (74 Ci/mmol) was from New England Nuclear Chemicals, GmbH, Dreieich, West Germany. WGA-Sepharose 6MB, Sepharose CL-6B, Sepharose 4B, and PD-10 columns with Sephadex G-25M were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden. WGA and agarose type I were from Sigma Chemicals Co., St. Louis, MO. Freund's complete and incomplete adjuvant were obtained from Difco Laboratories, Detroit, MI. Ready-Solv HP/b was from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Inhibition of Ad2 attachment by antiserum against HeLa cell plasma membranes

An antiserum raised against isolated plasma membranes was added at a 1:10 dilution to different concentrations of cells, ranging between 1×10^6 to 5×10^7 cells per ml. The cell-antiserum mixtures were incubated at either 4 or 37°C for 60 min. The cells were washed twice and ^3H -thymidine labeled virions were allowed to attach at a MOI of 340 for 45 min at 37°C for both series. The cell concentration during the viral attachment was 5×10^7 cells per ml. The inhibition of the viral attachment was highly dependent of the cell concentration and the temperature during the antiserum treatment (Fig. 1). The most pronounced impairment was at a cell concentration of 1×10^6 cells per ml and at 37°C . These conditions were employed in the experiments to follow.

The potency of the anti-total plasma membrane antiserum was also determined at different dilutions. Figure 5 shows that an inhibitory effect on the Ad2 attachment was discernible even at a high dilution of 1:150.

Effect of purified hexon and fiber antigen on Ad2 attachment

Virus-infected cells were labeled with ^{35}S -methionine late in the virus productive cycle, to ensure a preferential labeling of the viral structural proteins. The hexon and fiber antigen were isolated, by anion-exchange chromatography and further purified by gel filtration (hexon) or affinity chromatography (fiber). The purified antigens were added to cells and the adsorption was measured after 60 min at

30°C. When 5×10^5 fiber molecules per cell were incubated, around 12% were attached, whereas out of 2×10^6 added hexon molecules per cell, only 0.2% were adsorbed.

Subsequent viral attachment to cells, pretreated with either hexon or fiber antigen, was studied. Thus, virus attachment to cells was inhibited when cells were preincubated with the fiber antigen (Fig. 2). A dose-response relationship between the number of adsorbed fiber molecules per cell and the impairment of the Ad2 attachment was shown. On the other hand, pretreatment of cells with the hexon antigen did not significantly affect the viral adsorption.

In subsequent studies the purified viral antigens were immobilized to cyanogen bromide-activated Sepharose 4B, and used in intermediate agarose gels in the crossed immunoelectrophoretic analyses.

Crossed immunoelectrophoretic analyses

Plasma membrane proteins were solubilized with TX100 and subjected to electrophoretic separation in agarose gels, containing 0.5% TX100. All agarose gels contained TX100 to ensure proper solubility of the membrane proteins. Gel strips of the separated membrane proteins were cut out and placed facing an antiserum-containing gel and electrophoresed in a direction perpendicular to the first one. A reference pattern containing about 20 precipitates was obtained (Fig. 3A). To identify the cellular receptor protein(s), intermediate gels containing either of fiber antigen, hexon antigen or WGA immobilized to Sepharose 4B, were interposed between the gel of the separated membrane proteins of the first dimension and the antiserum-containing gel of the second dimension. One precipitate, designated I, of the reference pattern was reproducibly shown to diminish, when

fiber antigen was used in the intermediate gel (Fig. 3B and 4B). The specificity of the reaction was shown in analyses using intermediate gels containing only Sepharose 4B (Fig. 3C). These revealed immunoprecipitation patterns in close resemblance to or identical with the reference pattern. Immobilized hexon did not display any effect on the precipitation pattern (Fig. 4A), whereas WGA, even at relatively low concentrations, reduced the heights of one third of the around 20 precipitates, including the species affected by the fiber antigen (Fig. 4C). At higher concentrations of WGA in the intermediate gel the heights of the previously affected precipitates were further reduced (Fig. 4D).

TX100-solubilized membrane proteins were ^{125}I -labeled and analyzed by crossed immunoelectrophoresis. The precipitate, affected by immobilized fiber antigen, was cut out from 9 identical separations and eluted as described by Norrild et al. (23), and subsequently analyzed by SDS-PAGE. Autoradiography of the polyacrylamide gels, revealed a polypeptide with a molecular weight of 39,000 (not shown).

Antisera obtained against individual immunoprecipitates recovered from crossed immunoelectropherograms

a) Effect on Ad2 attachment

Individual precipitates, designated I and II (Fig. 3A), from 36 identical crossed immunoelectrophoreses were cut out, pooled and processed as described in Materials and Methods, before immunization into rabbits. After the fourth immunization, the rabbits were bled and recovered antisera were analyzed for inhibitory capacity on the viral attachment. Because of the poor inhibitory effect of these antisera (not shown), the rabbits were boosted with isolated total plasma membranes. The two antisera from the boosted rabbits were

shown to reduce subsequent Ad2 adsorption to various degrees (Fig. 5). A 1:10 dilution of the antiserum against the upper precipitate (designated II) gave the most pronounced effect, with a 52% inhibition of the viral attachment. This should be compared with the 46% inhibition by the pooled anti-total plasma membrane antiserum and the 18% inhibition obtained by the anti-precipitate I antiserum.

Crossed immunoelectrophoreses with the two antisera analyzed against TX100-solubilized plasma membrane proteins, demonstrated that both sera were polyspecific. Thus, five and seven immunoprecipitates were observed in the second dimension against anti-precipitate I and anti-precipitate II antiserum, respectively. Moreover, the anti-precipitate II antiserum contained antibodies directed against a membrane component, which was affected by fiber antigen immobilized to Sepharose. This protein species was identified as the constituent of immunoprecipitate I by means of the relative electrophoretic mobility and the morphology of the immunoprecipitate (not shown).

b) Immunoprecipitation in solution

To further characterize the antisera against immunoprecipitates I and II, these were used in immunoprecipitation analyses of ^{125}I -labeled TX100-solubilized membrane proteins as described in Materials and Methods. Antibodies and antigen-antibody complexes were desorbed from the immunosorbent in a 2% SDS-solution and analyzed by SDS-PAGE. Autoradiography of the polyacrylamide gels revealed a polypeptide pattern shown in Figure 6. The polypeptides of the immunoprecipitated proteins showed remarkable resemblance between the two antisera. The preimmune sera demonstrated weak background polypeptide patterns. Thus, in precipitates obtained with anti-precipitate II antibodies two polypeptides with molecular weights of 51,000 and 39,000 were particularly apparent. In the case with antibodies directed against precipitate I a unique 43,500 molecular weight polypeptide was revealed.

DISCUSSION

In most immunological methods, antibodies with known specificity are of absolute necessity. In this report isolated plasma membranes from HeLa cells were used as immunogen in rabbits, for production of a polyspecific antiserum. Because the cellular receptor sites for Ad2 are sparsely represented on the cells (16,32), rabbits were immunized over a long period (17 months) to ensure an immunological response even against the minor components of the membrane (6). Inhibition of the Ad2 attachment to cells, preincubated with the recovered antiserum, was apparent. The impairment was highly dependent of the cell concentration and the temperature at the step of the antiserum treatment of the cells. The cell concentration dependence is not surprising, since the dilution of the antiserum should be considered in relation to the total cellular volume. Axler and Crowell (3) have also reported corresponding results, concerning the inhibitory effect on the enterovirus attachment to HeLa cells in response to an anti-cellular antiserum.

The antiserum against total membrane proteins was used in crossed immunoelectrophoretic analyses of TX100-solubilized membrane proteins from HeLa cells. The analyses were performed in gels containing 0.5% TX100, to avoid undesirable effects due to insufficient solubility of the membrane proteins (5). Furthermore, 0.5% TX100 does not interfere with antigen-antibody interactions in agarose gels (9). In the described analyses a reference pattern of ca. 20 immunoprecipitates was reproducibly obtained. To identify the components of the cellular receptor sites for Ad2 in the precipitation pattern, the structural viral fiber and hexon antigens were purified and used in intermediate gels (2,31). In a separate study and before the immuno-

electrophoretic analyses, it was confirmed that the fiber antigen is the viral attachment protein as previously shown (28). Thus, the viral attachment to cells pretreated with this antigen was quantitatively impaired. Treatment of cells with the hexon antigen had no effect on the Ad2 adsorption. These observations were indirectly corroborated by the crossed immunoelectrophoretic analyses with intermediate gels, containing either of the viral antigens. One precipitate of the reference pattern was affected by the fiber antigen, and there was a close connection between the amounts of fiber antigen in the intermediate gel and the degree of reduction of the height of the precipitate designated I. Again, the hexon antigen did not display any effect on the precipitation pattern. On the other hand, immobilized WGA in the intermediate gel drastically changed the precipitation profile. Precipitate I disappeared even at rather low concentrations of WGA, thereby indicating a glycoprotein nature for this membrane component.

To obtain further information about the protein species with affinity for the fiber antigen, precipitates designated I and II were cut out from crossed immunoelectropherograms, and used as immunogens. Precipitate II was chosen because the reference pattern revealed this precipitate in close proximity to precipitate I. Recovered antiserum against precipitate II was initially meant as a "background" antiserum for the anti-precipitate I antiserum. The derived antisera indeed showed an inhibiting capacity on the viral attachment, but only after the animals were boosted with total plasma membranes. The fact that the antiserum against precipitate II was a more than thirty times as potent inhibitor as the anti-precipitate I antiserum, reflects the difficulties in the isolation of individual precipitates due to e.g. trapping and masking effects. The polyspecificity of the antisera, as

judged by crossed immunoelectrophoresis, further strengthen this observation, even though the plasma membrane-booster clearly have enhanced the immunological response against any contaminants of the precipitate preparations. One way to overcome the problem of poly-specificity, might be to cut out precipitates from electropherograms run with antibodies obtained from repeated immunizations in different animals, with such isolated immunoprecipitates (4). The antiserum with the pronounced inhibitory effect on the viral attachment, precipitated in immunoelectrophoretic analyses a membrane protein, which was affected by the presence of fiber antigen in the intermediate gel. The electrophoretic mobility of this protein and the morphology of the formed precipitate, strongly suggest that this precipitate is identical with the precipitate designated I.

Immunoprecipitations in solution of ^{125}I -labeled plasma membrane proteins were performed, with antisera against precipitates I and II. Two polypeptides with the apparent molecular weights of 39,000 and 51,000 were specifically immunoprecipitated by anti-precipitate II antiserum. The former polypeptide constitutes, together with the corresponding antibody, precipitate I, since direct elution of this precipitate revealed a polypeptide with identical molecular weight. We have earlier isolated a membrane component from HeLa cells with affinity both for WGA and complete Ad2 particles, and with a polypeptide molecular weight of 40,000 to 42,000 (32). This component is probably identical with the membrane protein constituting precipitate I.

Consequently, antibodies against at least one plasma membrane protein, have been shown to inhibit the Ad2 attachment to cells. The corresponding protein recognizes the fiber antigen, contains acetylated glucosaminy residues and has an apparent molecular weight of 39,000.

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FIGURE LEGENDS

Figure 1.

Effect of antiserum against total plasma membranes on the Ad2 attachment to HeLa cells. Different concentrations of cells, between 1×10^6 to 5×10^7 cells per ml, were incubated for 60 min at 4 or 37°C with a 1:10 dilution of an antiserum directed against isolated plasma membranes or a preimmune serum. The cells were subsequently washed twice with PBS and suspended at a density of 5×10^7 cells per ml, and ^3H -thymidine labeled Ad2 particles, at a MOI of 340, were allowed to attach to the cells at 37°C for 45 min. The viral attachment was determined and the specific inhibitory effect of the anti-total plasma membrane antiserum was calculated as described in the text. The data represent an experiment with an anti-total plasma membrane antiserum, from the final bleeding (compare Fig. 5), where open and solid circles represent antiserum incubations at 4 and 37°C , respectively.

Figure 2.

Ad2 attachment to HeLa cells pretreated with fiber and hexon antigen. To cells were added either 2×10^6 hexon molecules per cell or 1×10^5 to 5×10^5 fiber molecules per cell. After incubation at 3°C for 60 min, the number of adsorbed antigen molecules per cell was estimated as indicated in the Figure. For calculations, the molecular weights of 180,000 and 360,000 were used, for the native fiber and hexon antigen, respectively (8,12,30). ^3H -thymidine labeled Ad2 was added, at a MOI of 2,000, to the antigen-treated cells, and the viral attachment kinetics at 37°C was determined. In a control series cells were treated as described above, but without addition of viral antigens.

f; fiber, h; hexon

Figure 3.

Crossed immunoelectrophoretic analyses of TX100-solubilized plasma membrane proteins from HeLa cells. One hundred and twenty μg of total membrane proteins solubilized with TX100 was separated in the gel of the first dimension. Proteins in the gels were transferred quantitatively and electrophoresed against a gel, containing 20% (v/v) anti-total plasma membrane antiserum (denoted ab).

A. Typical reference pattern of immunoprecipitates. I and II indicate precipitates of further interest, and the gel marked with an asterisk is a visualization of the first dimension separation of membrane components after denaturation and staining of the proteins.

B. Precipitation pattern obtained in response to an intermediate gel (int) containing 4.5 μg of fiber antigen immobilized to Sepharose 4B. The analyzed membrane proteins were derived from ca. 2.6×10^7 cells, and the number of fiber molecules participating in the interaction with membrane components, constituting precipitate I, was ca. 4×10^{12} (dotted area). This means that about 150,000 fiber molecules per cell equivalent were engaged in the reaction (compare Fig. 2 and 4B).

C. Intermediate gel only containing the corresponding amount of Sepharose 4B as in B.

The electrophoreses were performed as described in the text.

Figure 4.

Crossed immunoelectropherograms of membrane proteins in the presence of different intermediate gels.

TX100-solubilized membrane proteins (100 μ g) were analyzed by crossed immunoelectrophoresis against a gel, containing 16.7% (v/v) anti-total plasma membrane antiserum. Intermediate gels, with different antigens immobilized to Sepharose 4B, were interposed between the gel of the first dimension and the antiserum-containing gel of the second dimension;

A. Intermediate gel, containing 24 μ g of hexon antigen. Considering the amounts of analyzed membrane proteins and the number of hexon molecules (5.5×10^{12}) in the area beneath precipitate I, 2.5×10^5 hexon molecules per cell equivalent were presented to the relevant membrane component.

B. Fiber antigen (2.2 μ g) in the intermediate gel. Analogous calculations as in A, yielded 90,000 fiber molecules per cell equivalent (compare Fig. 2 and 3B).

In C and D, 45 and 180 μ g of immobilized WGA, respectively, were used in the intermediate gels. Electrophoretic conditions were as outlined in the text.

Figure 5.

Effect of antisera against membrane components on the Ad2 attachment to HeLa cells. Different dilutions of antisera against total plasma membranes (pooled antiserum, ●—●), precipitate designated I (■—■), or precipitate II (▲—▲) were added to cells at a density of 1×10^6 cells per ml. The antiserum-cell mixtures were incubated at 37°C for 60 min, and the subsequent attachment of radioactively labeled virions was determined, as described in Fig. 1.

Figure 6.

SDS-PAGE of membrane proteins immunoprecipitated by antisera produced against precipitates I and II. TX100-solubilized membrane proteins were ^{125}I -labeled and immunoprecipitated with 5 or 25 μl of the immunoglobulin fraction of either anti-precipitate I antiserum or anti-precipitate II antiserum. Samples recovered as described in the text, were applied to each sample well in equal amounts of radioactivity (20,000 cpm). Nonspecific precipitation patterns were obtained in an analogous way with the immunoglobulin fraction from preimmune sera. A ^{14}C -amino acid-labeled preparation of Ad2 was used as a polypeptide marker with indicated molecular weights of the major structural polypeptides, as described by Anderson et al. (1). The arrow heads indicate polypeptides of 39,000 and 51,000 molecular weight. The electrophoretic separation was performed at 50V for 60 min and continued for an additional 160 min at 100V. After staining, the gel was dried under vacuum, and subsequently exposed to an X-ray film.

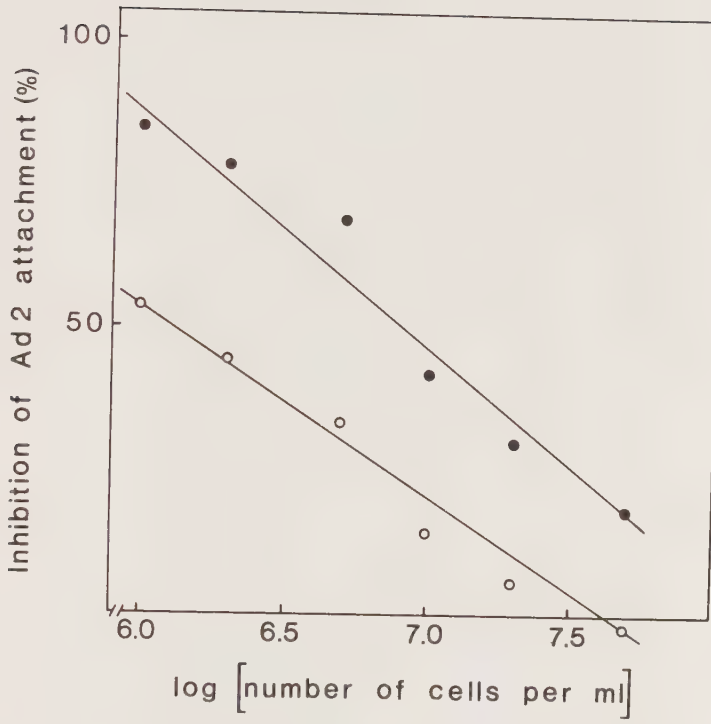


Figure 1.

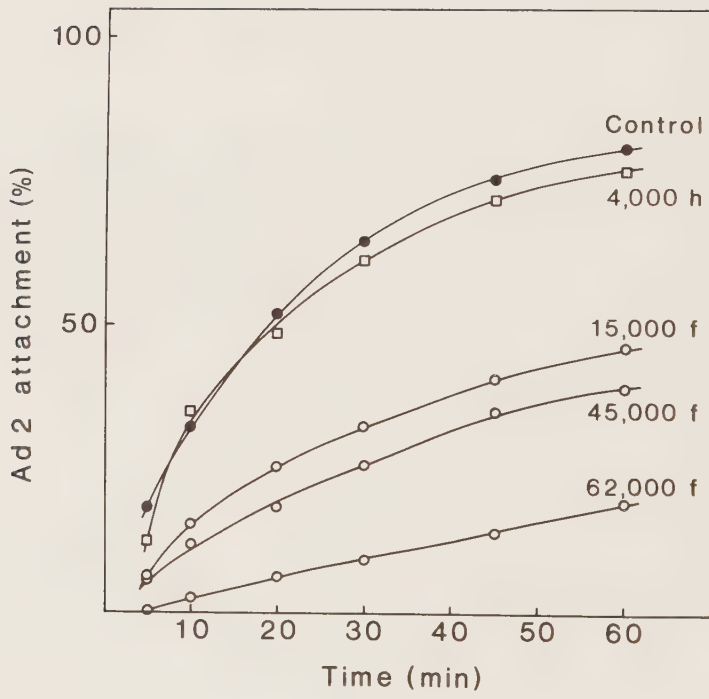


Figure 2.

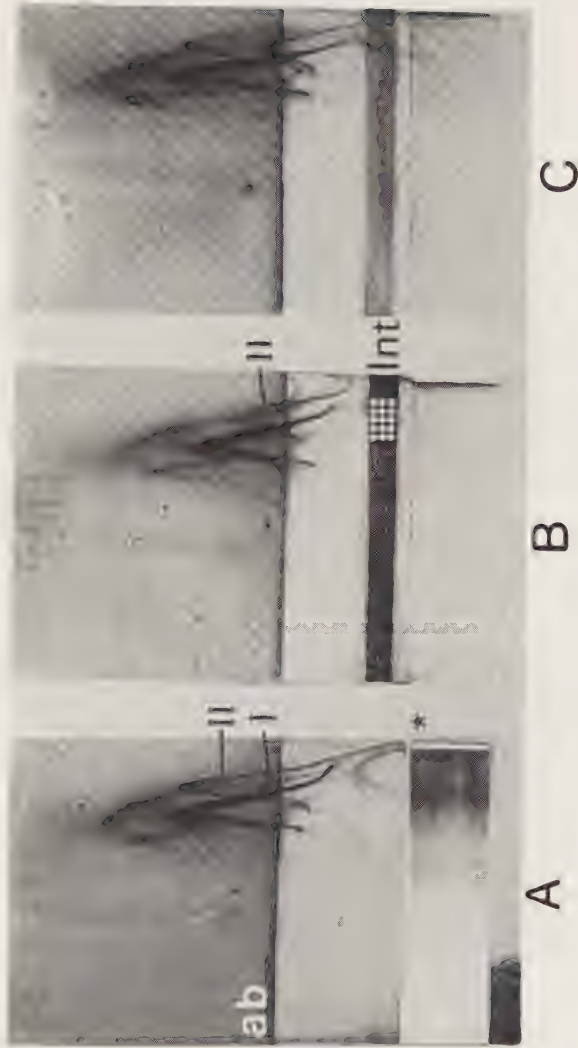


Figure 3A, B, and C.



Figure 4A, B, C, and D.

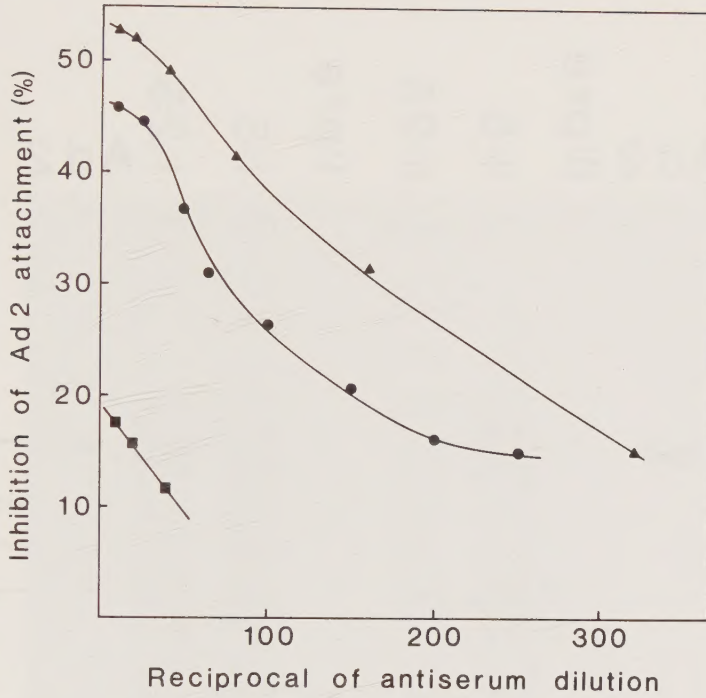


Figure 5.

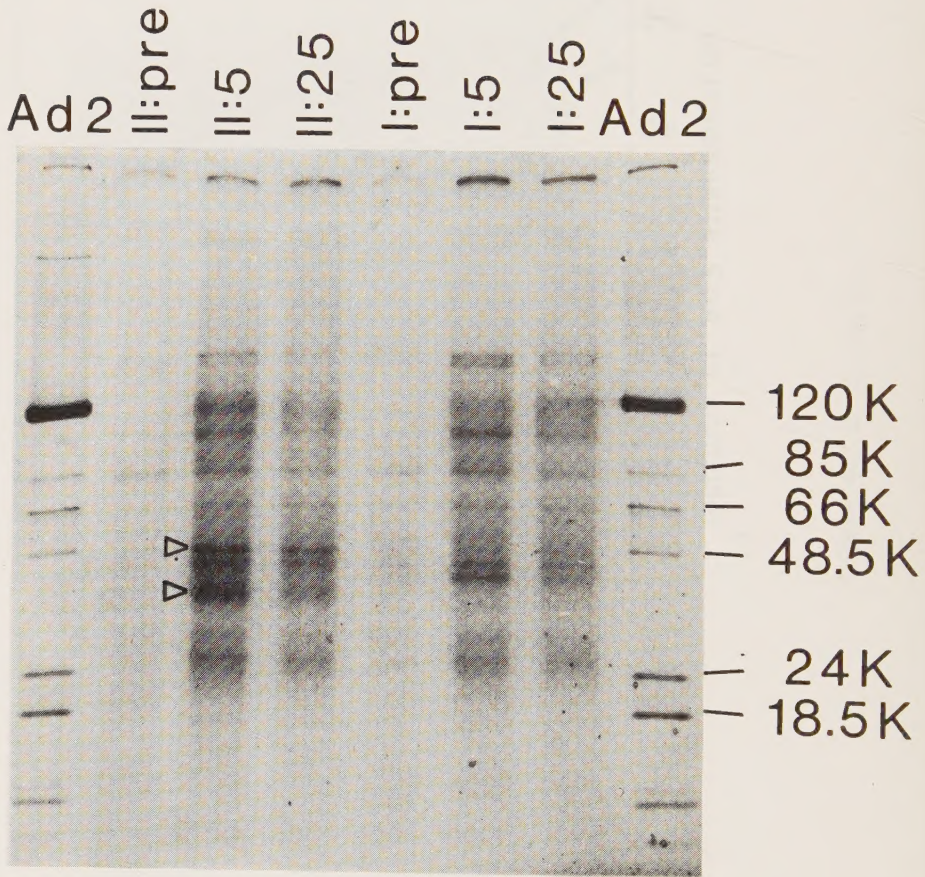


Figure 6.

